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# THERMAL DENATURATION OF TOBACCO MOSAIC VIRUS

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(Received for publication, November 29, 1939)

In recent studies (6, 7), the thermal inactivation of unpurified tobacco mosaic virus was shown to follow the course of a first order reaction. However, dilution of the plant juice containing the virus was found to result in an increase in the reaction rate. Moreover, the thermal inactivation point of this unpurified virus was observed to be between 90° and 93°, in contrast with the thermal inactivation point of about 75° reported for the purified virus by Stanley and Loring (9). In view of these observations, it was thought desirable to study the kinetics of the heat denaturation of purified virus under relatively well controlled conditions in order to learn whether or not there is any relationship between the inactivation and denaturation phenomena and to find, if possible, explanations of some of the observations just mentioned.

## EXPERIMENTAL

*Discussion of Experimental Method*—Most of the experiments reported were carried out on an old sample of virus purified by ammonium sulfate precipitation (8). In two experiments, a fresh sample of virus purified by differential centrifugation (11) was used. Except when otherwise designated, a few ml. of a cold concentrated solution of virus dissolved in 0.1 M phosphate buffer were added to at least 3 times their volume of hot 0.1 M phosphate buffer solution at the same pH value in a large test-tube immersed in a water bath held at the desired temperature,  $\pm 0.2^\circ$ . The virus solution in the bath was stirred constantly by mechanical means. The stirring and the adding of the virus to hot buffer were resorted to in order to reduce the time required

by the virus to reach the temperature of the bath. Samples were withdrawn from the tube periodically by means of a pipette and were immediately cooled by immersing in ice water. In a few tests, the virus was heated without agitation in a number of small tightly stoppered test-tubes immersed in the water bath or stored in oil baths in constant temperature ovens. Periodically one tube from each batch was cooled as described above. Under the conditions obtaining in this study, the denatured virus precipitates, even before cooling, in moderately large amorphous particles. This material was separated from the remaining soluble protein by spinning for a few minutes in a laboratory centrifuge. The concentration of the soluble material was then determined by the micro-Kjeldahl method.<sup>1</sup>

In addition to the errors inherent in the micro-Kjeldahl method, several limitations are encountered in determinations of this sort. A brief period is required for the virus in hot buffer to attain the temperature of the bath. Vigorous stirring of both the reacting solution and the bath tends to decrease this lag. Its effect becomes negligible if the first sample is withdrawn after the equilibrium temperature has been reached and if the initial virus concentration is ignored in calculations of the rate of the reaction. A second source of error is a consequence of the relatively high temperature coefficient of the denaturation reaction. Minor fluctuations in temperature may result in appreciable error. At the higher temperatures, the reaction proceeds so rapidly that samples must be taken at time intervals only slightly longer than the time required for the sampling process. Obviously, serious errors may result in this case. A third error should be expected in light of the finding of Bawden and Pirie (1) and of Stanley and Loring (9) that nucleic acid is split off from the virus upon heat denaturation.

Under the conditions of denaturation obtaining in this study it was found that essentially all of the carbohydrate, as measured by the orcinol method, remains soluble upon denaturation of about 90 per cent of the virus. From this observation it may be inferred that all or most of the nucleic acid of the denatured virus has remained in the supernatant liquid. The Kjeldahl method

<sup>1</sup> The authors wish to acknowledge the assistance of Mr. Martin Hanig, who performed the analyses reported in this paper.

should be expected, therefore, to give values for the protein concentration of the supernatant liquid which are somewhat too high. The error should progress from 0 at the beginning of the denaturation to the order of 50 per cent when the reaction has reached 90 per cent completion. It is possible to correct the experimental data by using the following equation,  $C = (C_1 - bC_0)/(1 - b)$ , where  $C$  is the actual concentration of virus protein in the supernatant liquid,  $C_1$  is the measured protein concentration,  $C_0$  is the initial concentration of the virus, and  $b$  is the fraction of the total nitrogen of the virus present in the nucleic acid. In the experiments here reported,  $C_0$  has an approximate value of 6 mg. per ml. in most cases, and 3 mg. per ml. in the remaining cases. According to Loring (4), tobacco mosaic virus contains about 5.2 per cent nucleic acid which has a nitrogen content of about 15.1 per cent. The factor  $b$  then becomes  $(5.2 \times 15.1)/(100 \times 16.6) = 0.047$ . Although it can be shown mathematically that, if the reactions are allowed to proceed only to about 80 per cent of completion, no serious error can result in the estimates of the reaction velocity constants due to the nucleic acid accumulation, the error does make it more difficult to decide upon the order of the reaction. Especially for this latter reason, the data reported in this paper are all corrected by means of the equation described above.

*Presentation of Data*—The results of eighteen denaturation experiments on purified tobacco mosaic virus carried out as described previously are summarized in Table I. All experiments were done with old, chemically purified viruses, except Experiments 17 and 18, in which ultracentrifugally isolated material was used. In Experiments 8, 11, and 16, the virus was heated without agitation in stoppered tubes instead of in the open tube with stirring. The initial concentrations of virus in the reacting solutions were about 6 mg. per ml. in most experiments and about 3 mg. per ml. in the others. The former are designated as initial dilutions of 1:1 and the latter as 1:2 in Table I. The pH values reported were measured at room temperature by means of a glass electrode on solutions prepared in the same manner from the same stock solutions as those being heated. The  $k$  values reported were calculated from the slopes of the straight lines best fitting the data when the natural logarithms of the corrected concentrations

## Virus Denaturation

TABLE I

*Heat Denaturation of Tobacco Mosaic Virus under Various Conditions*

Experiment No.	Temperature	Initial dilution	pH at room temperature	Time	Concentration of soluble protein		<i>k</i> (first order)
					Measured	Corrected	
	°C.			min.	mg. per ml.	mg. per ml.	min. <sup>-1</sup>
1	67	1:1	7.05	10	6.24	6.24	0.00424 ± 0.00015
				20	6.04	6.04	
				32	5.74	5.73	
				40	5.45	5.42	
				50	5.35	5.32	
				60	5.14	5.10	
				70	4.88	4.82	
				80	4.72	4.66	
2	69	1:1	7.05	15	5.23	5.19	0.00874 ± 0.00013
				30	4.52	4.45	
				45	3.95	3.74	
				60	3.51	3.39	
				75	3.14	3.00	
				90	2.83	2.68	
3	71	1:1	7.05	3	5.74	5.74	0.0472 ± 0.0016
				6	4.72	4.66	
				9	4.37	4.29	
				12	3.76	3.65	
				15	3.43	3.30	
				18	2.87	2.72	
				21	2.50	2.33	
				24	2.31	2.13	
4	73	1:1	7.05	3	4.44	4.37	0.198 ± 0.003
				6	2.63	2.46	
				9	1.59	1.37	
				12	0.98	0.73	
5	73	1:1	7.05	1	5.80	5.78	0.171 ± 0.001
				2	5.20	5.16	
				3	4.05	3.96	
				4	3.82	3.72	
				5	3.55	3.43	
				6	2.80	2.64	
				8	1.86	1.66	
				8	0.94	0.69	
6	74	1:1	7.05	2	4.66	4.60	0.311
				4	2.97	2.82	
				6	1.86	1.66	
				8	0.94	0.69	

TABLE I—*Continued*

Experiment No.	Temperature	Initial dilution	pH at room temperature	Time	Concentration of soluble protein		<i>k</i> (first order)
					Measured	Corrected	
	°C.			min.	mg. per ml.	mg. per ml.	min. <sup>-1</sup>
7	76	1:1	7.05	1	5.06	5.02	0.855
				2	2.63	2.47	
				3	1.15	0.91	
8*	60	1:2	7.05	460	2.72	2.70	(6.31 ± 0.43) × 10 <sup>-5</sup>
				1,440	2.48	2.46	
				1,880	2.60	2.58	
				2,860	2.41	2.38	
				5,750	1.97	1.92	
				8,600	1.69	1.63	
9	69	1:2	7.05	5	2.70	2.68	0.0354 ± 0.0045
				20	2.02	1.97	
				35	1.52	1.45	
				50	0.67	0.56	
				65	0.47	0.35	
10	71	1:2	7.05	3	2.46	2.43	0.0894 ± 0.0120
				6	1.86	1.80	
				9	1.11	1.02	
				12	1.18	1.09	
				15	0.84	0.73	
				18	0.88	0.78	
				21	0.51	0.39	
11*	71	1:2	7.05	0	3.16	3.17	0.0880
				7	1.75	1.69	
				14	1.01	0.91	
				21	0.62	0.50	
12	73	1:2	7.05	2	2.19	2.15	0.352 ± 0.029
				3	1.52	1.45	
				4	1.32	1.24	
				5	0.84	0.73	
				6	0.64	0.52	
13	78	1:1	6.10	10	5.20	5.16	0.087
				20	3.62	3.50	
				30	1.15	0.91	
14	79	1:1	6.10	6	4.99	4.98	0.121 ± 0.018
				9	4.55	4.48	
				12	3.64	3.52	
				15	2.87	2.72	
				18	1.48	1.26	
				21	1.08	0.84	

## Virus Denaturation

TABLE I—*Concluded*

Experiment No.	Temperature	Initial dilution	pH at room temperature	Time	Concentration of soluble protein		<i>k</i> (first order)
					Measured	Corrected	
	°C.			min.	mg. per ml.	mg. per ml.	min. <sup>-1</sup>
15	81	1:1	6.10	3	4.89	4.84	0.184
				6	3.34	3.26	
				9	1.92	1.72	
				12	1.18	0.94	
16*	71	1:1	5.77	5,320	5.70	5.68	(1.33 ± 0.22) × 10 <sup>-4</sup>
				22,600	3.54	3.42	
				44,200	3.37	3.24	
				68,680	2.70	2.54	
				90,280	1.75	1.54	
17†	69	1:1	7.05	10	5.52	5.50	0.0135
				75	2.46	2.28	
18†	73	1:1	7.05	1	5.77	5.75	0.201
				7	1.92	1.72	

\* Stopped tubes, no stirring.

† Ultracentrifugally isolated virus.

of undenatured virus were plotted against the time of the reaction. They are, therefore, first order reaction velocity constants expressed in units of reciprocal minutes. The justification for calculating first order constants will be considered presently.

*Order of Reaction*—As was shown by Chick and Martin (2), the heat denaturation reactions of hemoglobin and egg albumin are of the first order. The results of Wu and Ling (10), on the other hand, indicate that the surface denaturation reactions of methemoglobin and egg albumin are of the zero order. A reaction of this latter type progresses at a constant rate irrespective of the concentration of the reacting material, and it can be described by the simple differential equation,  $-dC/dt = k_0$ .  $C$  is the concentration of the undenatured material,  $t$  is the time, and  $k$  is the velocity constant. If the rate of the denaturation at any given instant is directly proportional to the concentration of the reactant at that instant, the reaction is of the first order and may be described by the equation,  $-dC/dt = k_1C$ . If two protein particles must come together and react before they can denature,

the process is a second order reaction which can be described by the equation  $-dC/dt = k_2C^2$ . If three particles must come together simultaneously before denaturation can occur, the reaction is of the third order and may be described by the equation,  $-dC/dt = k_3C^3$ . When these four equations are integrated, they may be written as follows: zero order,  $C = C_0 - k_0t$ ; first order,  $\log_e C = \log_e C_0 - k_1t$ ; second order,  $1/C = 1/C_0 + k_2t$ ; third order,  $1/C^2 = 1/C_0^2 + 2k_3t$ . In these equations,  $C_0$  is the initial concentration and  $t$  is the time interval from the beginning of the reaction. In each of the above equations, some

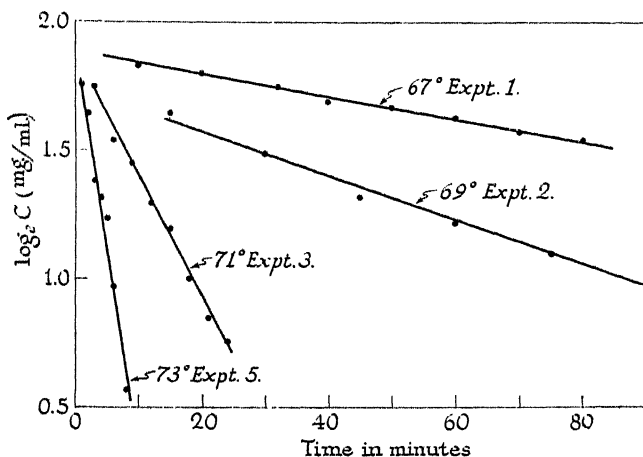


FIG. 1. Curves illustrating denaturation rates at several different temperatures of purified tobacco mosaic virus in 0.1 M phosphate buffer. (Initial concentrations about 6 mg. per ml., pH 7.05.)

function of the concentration is linearly related to the time, and the slope of the line in each case is a simple multiple of the velocity constant.

It may be seen in Fig. 1 that when  $\log_e C$  is plotted against  $t$  for the denaturation of the virus at several temperatures, the data fit straight lines fairly closely, indicating that heat denaturation of the virus may follow the course of a first order reaction. However, inasmuch as there is considerable error in each experimental point, it is also possible to fit straight lines to the data when either  $C$  or  $1/C$  is plotted against  $t$ . The data allow no doubt



that  $1/C^2$  is not a linear function of  $t$ . Although simple inspection reveals that the fit is usually best when  $\log_e C$  is plotted against  $t$ , the data have been treated statistically in order that the conclusion concerning the order of the reaction may be as sound as the data permit. For all the experiments reported in Table I in which more than four experimental points have been recorded, the best fitting straight lines were determined by the method of least squares for the cases in which  $C$ ,  $\log_e C$ , and  $1/C$  are considered as functions of  $t$ . The standard error of estimate was then calculated for each line according to the following equation, standard error =  $(\Sigma (Y - Y')^2) / ((N - 2) \Sigma (\bar{X} - X)^2)$ , when  $Y$

TABLE II

*Comparison of Errors Involved in Fitting Denaturation Data to Zero, First, and Second Order Equations*

Experiment No.	$\tau$ value		
	0 order	1st order	2nd order
1	26.26	29.06	26.35
2	10.65	21.29	42.23
3	14.52	29.75	16.19
5	13.01	12.50	6.34
8	12.68	14.63	14.60
9	11.12	7.94	4.40
10	5.55	7.45	4.57
12	8.60	12.24	6.15
14	13.66	6.89	4.25
16	4.39	6.02	4.63

is the calculated value of  $C$ ,  $\log_e C$ , or  $1/C$  as the case may be,  $Y'$  is the observed value,  $N$  is the number of observations,  $\bar{X}$  is the mean time, and  $X$  is the observed time. The slopes of the lines divided by their standard errors are summarized in Table II. The greater the magnitude of this statistic, designated here as  $\tau$  value, the better is the fit of the data to the straight line in question. It may be seen in Table II that in seven of the ten experiments thus analyzed the data favor first order over zero order equations and in nine of the ten cases first order over second order equations. Since there is no good reason to believe that the order of the reaction should be different at one temperature from that at another, the data taken as a whole make it appear probable

that the denaturation of the virus is a reaction of the first order. This probability is enhanced by the fact that there can be no doubt that the thermal inactivation of the virus is a reaction of the first order (7). However, an experiment subjected to more precise control was designed specifically to yield further information on this question.

The general plan of the experiment parallels that described previously, but it differs from that of the others in that a larger volume of protein was used and many more precautions were exercised than would have been practical for any considerable number of tests. The temperature was held at  $69.8^{\circ} \pm 0.045^{\circ}$ , with fluctuations completing one cycle every 40 seconds. Evaporation from the reacting tube was prevented by placing a layer of melted paraffin over the surface of the hot material. 5 ml. samples were withdrawn every 14 minutes for the first 196 minutes, and a final sample was taken after 250 minutes. A long stainless steel tube, which was allowed to remain in the reacting system, and a 5 ml. hypodermic syringe were used for the sampling. The reaction, in this case, was allowed to proceed to 97 per cent completion, greatly increasing the sensitivity of the test for the order of the reaction. This was made possible by completely eliminating the error due to nucleic acid accumulation in the supernatant liquid. Instead of analyzing the supernatant liquids directly after the denatured protein was removed, we denatured aliquots of them to completion by heating for  $\frac{1}{2}$  hour at  $100^{\circ}$ , and the flocculated protein, separated by centrifugation and filtration, was measured by the micro-Kjeldahl method. This procedure allows one to detect the amount of soluble protein capable of becoming insoluble upon heating, which is, of course, exactly what is wanted. The results of this experiment are shown in Table III. In Fig. 2,  $\log_e$  of the concentration of coagulable soluble protein is plotted against time. It is evident that the reaction may be described by the first order equation. According to the method of analysis of the data used in the less sensitive experiments,  $\tau$  values for zero, first, and second order equations of 12.7, 68.6, and 7.6, respectively, were obtained. This result is overwhelmingly in favor of the reaction being of the first order.

*Degree of Reproducibility*—The degree of reproducibility of the

TABLE III

*Heat Denaturation of Tobacco Mosaic Virus (6 Mg. per Ml.) in 0.1 M Phosphate Buffer at pH 7 and 69.8°*

Time	Concentration of soluble, coagulable protein	Time	Concentration of soluble, coagulable protein
<i>min.</i>	<i>mg. per ml.</i>	<i>min.</i>	<i>mg. per ml.</i>
0	5.91	112	1.13
	5.96		1.16
14	4.20	126	0.84
	4.37		0.91
28	3.34	140	0.76
	3.29		0.81
42	2.84	154	0.69
	2.89		0.66
56	2.42	168	0.51
	2.42		0.55
70	2.05	182	0.43
84	1.65		0.45
	1.70	196	0.40
98	1.38		0.38
	1.38	250	0.14
			0.13

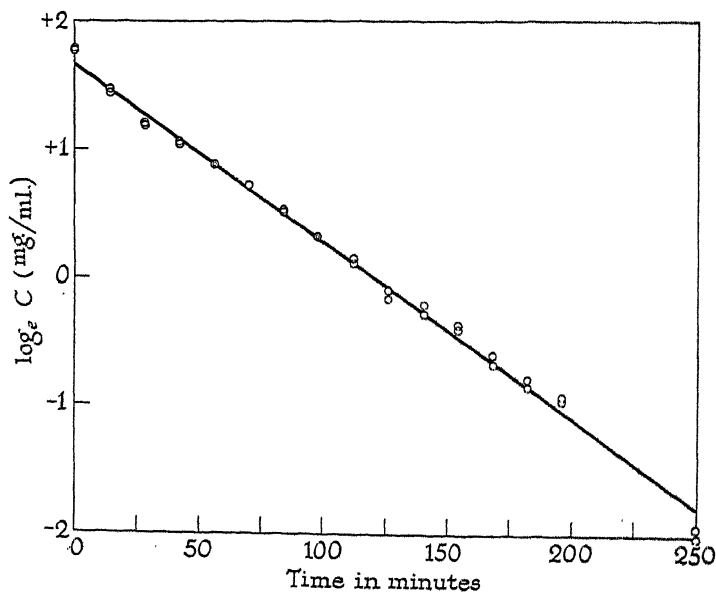


FIG. 2. Data of Table III plotted according to a first order equation

rate of denaturation of a given virus preparation may be estimated by comparing the velocity constants  $2.0 \times 10^{-1}$  and  $1.7 \times 10^{-1}$  calculated for Experiments 4 and 5 (Table I) on chemically isolated material at  $73^\circ$  and pH 7.05. The extent to which the denaturation rates are independent of the methods of virus preparation may be judged by comparison of the  $k$  value of Experiment 17 ( $13.5 \times 10^{-3}$  at  $69^\circ$ ) with that of Experiment 2 ( $8.7 \times 10^{-3}$  at  $69^\circ$ ) and by comparing the  $k$  value of Experiment 18 ( $2.0 \times 10^{-1}$  at  $73^\circ$ ) with those of Experiments 4 and 5 ( $2.0$  and  $1.7 \times 10^{-1}$ , respectively, at  $73^\circ$ ). Experiments 17 and 18 were conducted with recently ultracentrifugally isolated material, and Experiments 2, 4, and 5 with old, chemically purified virus. Evidently the rate of heat denaturation is not influenced by the differences between chemically and ultracentrifugally purified virus, which are reflected by the higher degree of aggregation and the lower specific infectivity of the former (5). The effect of stirring the reacting material may be seen by comparing the  $k$  value  $8.9 \times 10^{-2}$  of Experiment 10, carried out in the usual way, with the value  $8.8 \times 10^{-2}$  of Experiment 11 in which the virus was denatured without being stirred in several small stoppered test-tubes. A true picture of the reproducibility of this denaturation may be gained by examining the points, corresponding to the experiments mentioned above, lying near the lower curve of Fig. 3. The dimensions of the points on this curve are fair estimates of the error inherent in each determination. From all of these comparisons it may be concluded that the rate of denaturation of the virus is a reproducible property of the material.

*Temperature Coefficient*—Arrhenius proposed the equation  $d \log_e k / dT = E/RT^2$ , where  $T$  is the absolute temperature,  $E$  is a constant usually identified as the energy of activation, and  $R$  is the gas constant. This equation may be integrated and expressed in the form,  $\log_e k = \text{constant} - (E/RT)$ . By plotting  $\log_e k$  against  $1/T$  one should obtain a straight line of slope  $-E/R$ . It may be seen in Fig. 3 that when the logarithms of the  $k$  values obtained at pH 7.05 are plotted against  $1/T$  the points group fairly nicely about two straight lines with essentially equal slopes. The upper line is drawn to fit the data for systems whose initial concentrations were about 3 mg. per ml. and the lower curve is drawn to fit data for systems whose initial concentrations were

about 6 mg. per ml. The slope of these curves,  $-E/R$ , is equal to about  $-7.65 \times 10^4$ , and from this it may be estimated that  $E$ , the energy of activation, is about 153,000 calories per mole. This value is of the order of magnitude frequently encountered in protein denaturations.

*Effect of Dilution*—By comparing the  $k$  value reported in Table I obtained from Experiment 9 with those obtained from Experiments 2 and 17, those of Experiments 10 and 11 with that of Experiment 3, and that of Experiment 12 with those of Experiments

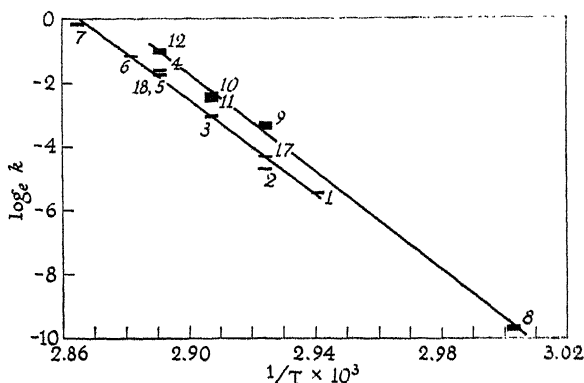


FIG. 3. Curves obtained when the velocity constants of the denaturation of tobacco mosaic virus at pH 7.05 are plotted as functions of absolute temperature according to the Arrhenius equation: upper curve for systems with initial virus concentration of about 3 mg. per ml.; lower curve for systems with initial virus concentration of about 6 mg. per ml. The numbers near the points on the curves represent experiment numbers corresponding to those recorded in Table I.

4, 5, and 18, it may be seen that virus solutions in which the initial concentration was about 6 mg. per ml. denature approximately half as fast as those in which the initial virus concentration was about 3 mg. per ml. By examination of Fig. 3 it may be seen that the straight line fitted by the method of least squares to the data for the dilute solutions is displaced from the one fitted to the data for the concentrated solutions by approximately  $\log_e 2$ . This increase in reaction rate upon dilution parallels the similar finding for the case of unpurified virus (7). As may be seen in Fig. 2 of the paper referred to, a 1:20 dilution with water of the plant

juice containing virus resulted in approximately a 20-fold increase in the rate of inactivation. Since there is a possibility that slight changes in pH accompanied the dilution in this latter case, it is impossible to say just how much of the observed increase in velocity is actually the effect of lowering the virus concentration. This uncertainty, however, does not obtain in the case of the purified virus. A possible explanation of the inverse relationship between initial virus concentration and reaction rate is that some grouping within the virus is acting as a negative catalyst or an inhibitor for the denaturation.<sup>2</sup>

*Effect of Changing the pH*—An examination of Experiments 13, 14, and 15 (Table I) reveals that at pH 6.10 denaturation takes place at a rate comparable to that at pH 7.05 only when the temperature is 7° or 8° higher. A comparison of Experiment 16 carried out at pH 5.77 at 71° with Experiment 3 carried out at pH 7.05 at 71° shows that there is a 3500-fold increase in the rate of denaturation at the higher pH over that at the lower one. It would appear that the reaction rate varies inversely with something like the third power of the hydrogen ion concentration,<sup>3</sup> being greater in more alkaline and less in more acid solutions. In this respect, the virus differs from those proteins which show a minimum rate of denaturation at the neutral point. As was pointed out previously, the thermal inactivation point of purified virus is about 75°, whereas that of the unpurified material in plant juice is between 90° and 93°. The acidity of expressed plant juice is generally about pH 5.5 and the studies on purified virus were carried out at acidities near pH 7 (1, 9). In view of the findings of the present study, these facts alone could account

<sup>2</sup> This effect of dilution may also be considered in terms of the concepts of absolute reaction velocities (see La Mer (3)). It may be calculated that the entropy of activation in initially dilute virus solutions is greater than in initially more concentrated solutions. Taking into account such factors as ionization would not change the magnitude of the difference in entropies of activation. However, the physical significance of this difference and its relationship to initial virus concentration may be, at best, but vaguely understood.

<sup>3</sup> This result would indicate that a dissociation of protons is one of the stages of the activation process, in keeping with the considerations of Steinhardt (see La Mer (3)). However, the experiments here reported were not designed to yield critical information on this issue, and no quantitative interpretation is warranted.

for at least a great part of the difference in stability between purified virus and virus in plant juice.

*Nature of Reaction*—Denaturation of a protein may be the result of a series of reactions. Studies of the sort reported here measure only the velocity of the process as a whole, a velocity likely to be that of the slowest reaction of a series. Specifically, in the case under consideration, the velocity of the slowest reaction in the series which leads to changes sufficient to produce precipitates of denatured protein is measured. This reaction is not identical with the thermal inactivation of the virus. The

TABLE IV  
*Infectivity of Protein Remaining Soluble after Heating*

Temperature	Time heated	Soluble protein remaining	Soluble protein used for inoculation	No. of lesions on 20 half leaves	No. of lesions on opposite halves, control at $10^{-5}$ mg. per ml.	Estimated infectivity of soluble protein
$^{\circ}\text{C.}$	min.	mg. per ml.	mg. per ml.			per cent
69	10	5.50	$10^{-5}$	183	398	19
			$10^{-4}$	557	404	
			$10^{-3}$	1493	184	
69	75	2.28	$10^{-5}$	129	453	9
			$10^{-4}$	511	566	
			$10^{-3}$	1176	329	
73	1	5.75	$10^{-5}$	233	885	16
			$10^{-4}$	805	566	
			$10^{-3}$	1573	828	
73	7	1.72	$10^{-5}$	82	592	4
			$10^{-4}$	400	595	
			$10^{-3}$	1062	713	

data presented in Table IV, which may be identified with Experiments 17 and 18 of Table I, were obtained by denaturing the virus for short and longer periods of time at two different temperatures, measuring the concentrations of the soluble protein, and comparing the infectivities of these on a concentration basis with untreated virus, with the bean as a test plant. Although estimates of the infectivity obtained by the method indicated in Table IV are not sufficiently accurate to allow calculation of the ratios between the rates of inactivation and denaturation, the data serve to point out that inactivation is a considerably faster

process than denaturation measured by precipitation. In all likelihood, inactivation of the virus is one of the earlier reactions in a series which finally leads to denaturation. It apparently is not the reaction of limiting velocity. The energy of activation calculated for the denaturation is of the same order of magnitude as that calculated for the thermal inactivation at the higher temperatures (7), indicating that the inactivation is probably due to a reaction similar to that which controls the denaturation rate.

#### SUMMARY

The thermal denaturation of tobacco mosaic virus was found to be a readily reproducible phenomenon and was shown to be a reaction of the first order. The energy of activation of the reaction in 0.1 M phosphate buffer at pH 7 was calculated to be about 153,000 calories per mole. The rate of the reaction at a given temperature was found to be strongly influenced by changes in hydrogen ion concentration, the reaction progressing more rapidly in more alkaline and less rapidly in more acidic solutions. The reaction rate was found to vary inversely with the initial concentration of virus, being approximately twice as great in systems with an initial concentration of 3 mg. per ml. as in solutions with an initial concentration of 6 mg. per ml. Thermal inactivation of virus was found to proceed more rapidly than thermal denaturation measured by precipitation.

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# PROPERTIES OF THE FILTRATE FACTOR OF THE VITAMIN B<sub>2</sub> COMPLEX, WITH EVIDENCE FOR ITS MULTIPLE NATURE\*

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The material herein reported deals with an attempt at concentration of the "filtrate factor" portion of the vitamin B<sub>2</sub> complex. This fraction has been variously termed filtrate factor (1-3), yeast filtrate factor (4-6), liver filtrate factor (7, 8), rat growth factor (9, 10), factor 2 (1, 3), etc.

The original filtrate factor included the chick antidermatitis factor but Woolley, Waisman, and Elvehjem (11) have prepared concentrates highly active for the chick but inactive for the rat.

Woolley *et al.* (12) and Jukes (13) have shown that the "chick antidermatitis factor" is probably identical with pantothenic acid, a substance necessary for yeast growth, the chemical nature of which has been extensively investigated by Williams and co-workers. But Hoffer and Reichstein (2) have also indicated that the rat filtrate factor is probably pantothenic acid, as the result of experiments in which growth was stimulated by  $\beta$ -alanine (a constituent of pantothenic acid) when administered to rats on a filtrate factor-deficient diet. Growth was likewise stimulated by

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a purified ether extract of a filtrate factor preparation. The residue after ether extraction produced a definite, although not as marked, an increase in growth.

El Sadr and coworkers (8) have found that  $\beta$ -alanine does not replace the liver or yeast filtrate factor and believe that the filtrate factor has at least three components.

Subbarow and Hitchings (9, 10) isolated a rat growth factor from a liver concentrate, employing the method used by Williams and coworkers (14) in the purification of pantothenic acid. The preparation was active when fed at a level of 8 mg. weekly and was unstable to acid and alkali.

Finally Oleson, Woolley, and Elvehjem (15) have recently found that crude preparations of pantothenic acid stimulated growth in animals fed a filtrate factor-deficient diet, but not to the same extent as did a liver filtrate. The pantothenic acid could be inactivated with NaOH. In addition to the alkali-labile factor (probably pantothenic acid), the rat required an additional factor (factor W of Frost and Elvehjem (16, 17)) that was present in a liver extract or in crude filtrate preparations. Factor W is not soluble in ether (17).

#### EXPERIMENTAL

The growth of female rats was taken as an indication of filtrate factor activity and color of the fur was noted, as Morgan, Cook, and Davison (18) and Lunde and Kringstad (19) had reported a graying of the pelage in rats on a filtrate factor-deficient diet.

The diet used was as follows: extracted casein (20) 24, salts (McCollum No. 185 (21)) 4, fresh lard 3, cod liver oil (Squibb) 2, sucrose 67.

Female rats were placed at weaning (21 days) on the above diet supplemented with 15 micrograms of thiamine six times weekly. At the end of 4 weeks all animals had attained a constant weight. They were then segregated into groups so that each litter was represented by one control. Six animals were used in each assay (unless otherwise noted). All animals were then given 6 times weekly, in addition to the thiamine, 20 micrograms of riboflavin and a wheat germ eluate (as a source of vitamin B<sub>6</sub>) equivalent to 1.5 gm. of defatted wheat germ.<sup>1</sup>

The results of the assays are presented in Table I. The gain in

<sup>1</sup> By assay the equivalent of 1.0 gm. produced the same gain as did 15 micrograms of crystalline vitamin B<sub>6</sub> (Merck).

TABLE I  
*Potency of Filtrate Factor Preparations from Various Source Materials,  
When Fed to Rats*

Preparations	Equiv- alent fed 6 times weekly	Solids fed 6 times weekly	Gain in weight above controls		Graying of rats noted	Growth- pro- moting activ- ity
			30 days	56 days		
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		
Hawaiian cane	0.58	0.475	53	100	1 (slight) out of 5	++
molasses	1.16	0.950	70	108	None	++
10A	3	0.121	4	14	4 out of 5	-
10B	3	0.310	54	71	None	++
11A	3	0.246	54	72	"	++
12A	2	0.630	21	39	5 out of 6	+
14A	3	0.024	54	75	None	++
14B	3	0.600	44	67	1 out of 4	++
14C	1	0.007	12	27	2 " " 5	+
30F	3	0.028	30	60	None	++
30G*	3	0.025	0		3 out of 3	-
30H†	3	1.600	19	58	3 " " 3	++
31B	3	0.013	47	50	None	++
31C	3	0.088	24	34	5 out of 6	+
36C	3	0.012	22	42	1 " " 4	++
Rice bran extract	0.65	0.450	59	97	None	++
(Galen B)	1.30	0.900	83	116	"	++
9B	3	0.008	53	74	"	++
29A‡	16	0.006	27	31	1 out of 1	+
29B‡	16	0.002	0		1 " " 1	-
29C†	16	0.003	48	56		++
32A	3	0.008	28	40	None	+
92% ethanol liver	1	0.886	126	145	"	++
extract§						
19A	5	0.150	59	84	"	++
20A	10	0.017	68		"	++
24A	5	0.010	56		"	++
35A	10	0.040	28	48	"	++
37A*	20	0.008	3	0	2 out of 3	-
37B*	20	0.010	2	7	2 " " 2	-
37C†	10	0.015	60	74	1 " " 5	++
Brewers' yeast	0.25		41	81	3 " " 3	++
	0.50		52	83	6 " " 6	++
					(3 slight)	
	1.00		91	121	1 out of 4	++
Brewers' yeast¶	0.50		54	105	5 (slight) out of 6	++
Whole milk powder**	1.00		16	23	3 out of 6	+

- denotes a weight increment in 56 days of less than 15 gm. above the controls; + an increment of 15 to 40 gm.; ++ an increment above 40 gm.

\* Three rats in the group.

† Five rats in the group.

‡ Two rats in the group.

§ Eli Lilly and Company.

|| Vitamin Food Company, Inc.

¶ Yeast Foam powder, Northwest-  
ern Yeast Company.

\*\* Golden State Company, Ltd.

weight of the controls in each group was subtracted from the gain in weight of the animals receiving the test materials. The presence or absence of graying in solid colored and hooded animals is also noted.

Cane molasses, an aqueous rice bran extract (Galen B), and a 92 per cent alcohol-soluble extract of liver (Lilly) were employed in the concentration of the filtrate factor.

### *Molasses Preparations*

*Preparation 10A*—To 1200 gm. of molasses, methyl alcohol was added until no further precipitation occurred (3 to 4 liters) (the insoluble fraction weighed 408 gm.). To the filtrate an equal volume of acetone was added (the insoluble fraction weighed 249 gm.). The filtrate was freed from methanol and acetone. The residue was taken up in 4 liters of  $H_2O$  and shaken with 1000 gm. of fullers' earth for 1 hour. The filtrate was distilled to dryness and was then dissolved in methanol. Ethanol was added until no further precipitation occurred. The insoluble fraction weighed 48.3 gm. This was dissolved in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of original molasses; solids in 1 cc. = 121 mg.; inactive. Graying noted in four out of five rats.

*Preparation 10B*—The filtrate from the above was freed from methanol and ethanol. The solids amounted to 124 gm. This was diluted with 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 310 mg.; active. No graying noted among the rats.

*Preparation 11A*—A concentrate prepared as was Preparation 10B containing 410 gm. of solids was acidified to pH 1 to 2 with HCl and was extracted five times with isoamyl alcohol, 250 cc. being used for each extraction. The vitamin in the isoamyl alcohol fraction was extracted with 1 per cent NaOH, which was then neutralized with HCl. The solid residue (18.4 gm.) was taken up in ethanol and the separated NaCl filtered. The solid residue from the filtrate was taken up in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 46 mg.; active. No graying observed among the rats.

*Preparation 12A*—The residue after extraction with isoamyl alcohol contained 380 gm. of solids which was diluted with 600 cc. of  $H_2O$ . 1 cc.  $\approx$  2 gm. of molasses; solids in 1 cc. = 630 mg.; somewhat active. Graying noted in five out of six rats.

*Preparation 14A*—A concentrate similar to Preparation 10B from 1500 gm. of molasses containing 320 gm. of total solids was dissolved in 1 liter of  $H_2O$  and acidified with  $HCl$  to pH 4. 20 gm. of norit A were added and the mixture was stirred for 1 hour, then filtered, and the norit adsorbate was eluted with a methyl alcohol-pyridine mixture (4:1). The eluate was freed of solvent (solids 12 gm.) and was diluted with 500 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids 1 cc. = 24 mg.; active. No graying observed among the rats.

*Preparation 14B*—The filtrate from the above norit adsorption containing 300 gm. of solids was concentrated to 500 cc. 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 600 mg.; active. Graying noted in one out of four rats.

*Preparation 14C*—A concentrate prepared as was Preparation 10B from 600 gm. of molasses and containing 173 gm. of solids was dissolved in 600 cc. of  $H_2O$  and saturated with  $NaCl$ . After being acidified with  $HCl$  to pH 2, it was extracted five times with butyl alcohol (with 200 cc. for each extraction).

The combined extracts were distilled to dryness and the solids of 4.2 gm. were dissolved in 600 cc. of  $H_2O$ . 1 cc.  $\approx$  1 gm. of molasses; solids in 1 cc. = 7 mg.; somewhat active. Graying noted in two out of five rats.

*Preparation 30F*—The methyl alcohol-soluble fraction from 1200 gm. of molasses (600 gm.) was dissolved in 1600 cc. of  $H_2O$  and adsorbed on 400 gm. of fullers' earth. The filtrate was acidified with  $HCl$  to thymol blue. It was then extracted with ether in a continuous extractor for 72 hours. 14.6 gm. of solids were extracted and dissolved in a small volume of methyl alcohol (about 50 cc.). A small amount of  $H_2O$  was added and the precipitate filtered off. The filtrate was freed of methyl alcohol and the volume adjusted to 400 cc. Total solids 11.2 gm.; 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 28 mg.; active. No graying of the rats observed.

*Preparation 30G*—The residue from the ether extraction was reextracted for 88 hours. The solids of 9.8 gm. so extracted were taken up in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 25 mg.; inactive. Graying noted in three out of three rats.

*Preparation 30H*—After extraction of Preparations 30F and 30G the residue was neutralized with  $Na_2CO_3$  and taken up in 800

cc. of  $H_2O$ . Solids, 640 gm. 2 cc.  $\approx$  3 gm. of molasses; solids in 2 cc. = 1.60 gm.; active. Graying noted in three out of three rats.

*Preparation 31B*—The methyl alcohol-soluble fraction from 4000 gm. of molasses (2000 gm.) was dissolved in 10 liters of  $H_2O$  and acidified to pH 2 and shaken with 1400 gm. of norit A. The norit A adsorbate was eluted with 90 per cent ethanol. The total solid eluted was 140 gm. which was taken up in 1600 cc. of  $H_2O$ . It was acidified to blue with Congo red and extracted with ether in the continuous extractor for 100 hours. The solids extracted were 20.45 gm., of which 17.54 gm. were  $H_2O$ -soluble. 5.25 gm. of preparation were dissolved in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 13 mg.; active. No graying among the rats observed.

*Preparation 31C*—The residue from the ether extraction of Preparation 31B equivalent to 1500 gm. of molasses was taken up in 500 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 88 mg.; somewhat active. Graying noted in five out of six rats.

*Preparation 36B*—6 gm. of Preparation 31B and 6 gm. of semicarbazide hydrochloride were dissolved in 60 cc. of  $H_2O$  and 8.4 gm. of sodium acetate were added. This was allowed to stand for 24 hours. No sparingly soluble derivative was formed. This preparation was not fed.

*Preparation 36C*—6 gm. of a concentrate like Preparation 31B were dissolved in 16 cc. of dry pyridine and 3 cc. of benzoyl chloride were added and the mixture was allowed to stand for 24 hours. No sparingly soluble derivative was formed.  $H_2O$  was added and the benzoyl chloride was hydrolyzed to benzoic acid, which was filtered off. The filtrate was freed of pyridine under reduced pressure and diluted to 500 cc. 1 cc.  $\approx$  3 gm. of molasses; solids in 1 cc. = 12 mg.; active. Graying noted in one out of four rats.

### *Rice Bran Preparations*

*Preparation 9B*—A concentrate from an aqueous rice bran extract was prepared as was molasses Preparation 10B and extracted with isoamyl alcohol as in the case of Preparation 11A and adsorbed on norit A and eluted as in the case of Preparation 14A. The eluate contained 14.4 gm. of solids. 3.1 gm. of this preparation were dissolved in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  3 gm. of rice bran

extract; solids in 1 cc. = 8 mg.; active. No graying observed among the rats.

*Preparation 29A*—3.8 gm. of Preparation 9B were dissolved in 50 cc. of acetic anhydride. 2 cc. of pyridine were added and the solution was heated for 1 hour on the steam bath. Excess reagents were removed by distillation. The acetylated mixture was dissolved in ether and extracted with  $H_2O$ . The  $H_2O$  solution was acidified and reextracted. The  $H_2O$  extract containing 0.9 gm. of solids was dissolved in 150 cc. of  $H_2O$ . 1 cc.  $\approx$  16 gm. of original rice bran extract; solids in 1 cc. = 6 mg.; somewhat active. Graying noted in the one colored rat on the sample.

*Preparation 29B*—The ether phase after removal of the solvent was 2.52 gm. This was distilled in a molecular still at  $10^{-5}$  mm. of Hg pressure at  $100^\circ$  for 11 hours. 0.843 mg. was distilled, which was neutralized with dilute NaOH. 673 mg. of benzylthiuronium chloride were dissolved in 4.5 cc. of ethanol and added to the hot alcoholic solution of the concentrate. As no solids separated, the ethanol was distilled off and the residue taken up in a small amount of acetone. Insoluble material was filtered off and the clear filtrate was allowed to stand for 24 hours at  $4^\circ$ . 465 mg. of material crystallized (m.p.  $130-133^\circ$ ). The crystals were dissolved in a few cc. of methyl alcohol, and about 20 cc. of  $H_2O$  were added. The solution was acidified to blue with Congo red and extracted with ether. The residue (172 mg.) from the ether extract was taken up in ethanol and hydrolyzed with gaseous  $NH_3$ . The product on hydrolysis was crystalline. Total solids, 150 mg. This substance was dissolved in 75 cc. of  $H_2O$ . 1 cc.  $\approx$  16 gm. of rice bran extract; solids in 1 cc. = 2 mg.; inactive. Graying noted in the one colored rat on the sample.

*Preparation 29C*—The residue from the filtrate after filtration of the acetone-insoluble material in Preparation 29B was 500 mg. This product on hydrolysis weighed 479 mg. and was taken up in 150 cc. of  $H_2O$ . 1 cc.  $\approx$  16 gm. of molasses; solids in 1 cc. = 3 mg.; active. No colored rats were fed the sample.

*Preparation 32A*—3.8 gm. of Preparation 9B were dissolved in 25 cc. of 1 N NaOH and heated on the steam bath for 1 hour. After neutralization, the volume of the solution was adjusted to 400 cc. 1 cc.  $\approx$  3 gm. of rice bran; solids in 1 cc. = 8 mg.; somewhat active. No graying among the rats.



*Liver Preparations*

Lilly's 92 per cent alcohol-soluble extract was used.

*Preparation 19A*—552 gm. of the Lilly preparation equivalent to 2500 gm. of fresh liver were dissolved in  $H_2O$  and adsorbed on fullers' earth. The filtrate was acidified and extracted with ether in a continuous extractor for 37 hours. 119 gm. of solids were extracted. The residue from this ether extract was extracted with benzene and chloroform. 23 gm. were extracted with benzene and 9 gm. with chloroform. The residue was again extracted with ether in a separatory funnel. 8.5 gm. of solids were extracted by this process. From the second ether extract, needle-like crystals were obtained which melted at  $120^\circ$ . The residue, which after extraction weighed 75 gm., was taken up in 500 cc. of  $H_2O$ . 1 cc.  $\approx$  5 gm. of fresh liver; solids in 1 cc. = 150 mg.; active. No graying observed among the rats.

*Preparation 20A*—The residue after the extraction of Preparation 19A was again extracted for 24 hours. 4.3 gm. of solids were extracted, which were taken up in 250 cc. of  $H_2O$ . 1 cc.  $\approx$  10 gm. of liver; solids in 1 cc. = 17 mg.; active. No graying of rats noted.

*Preparation 24A*—A concentrate from 552 gm. of Lilly's liver after extraction with ether in a continuous extractor for 92 hours containing 80 gm. of solids was acidified and adsorbed on 250 gm. of norit A. The adsorbate was eluted with 90 per cent ethyl alcohol. The solids weighing 5.2 gm. were taken up in 500 cc. of  $H_2O$ . 1 cc.  $\approx$  5 gm. of liver; solids in 1 cc. = 10 mg.; active. No graying among the rats was observed.

*Preparation 35A*—A concentrate prepared by precipitation of inert material with methyl alcohol and subsequent adsorption on fullers' earth and containing 176.5 gm. of solids was acetylated. The acetylated mixture which contained 20 gm. of solids was taken up in 500 cc. of  $H_2O$ . 1 cc.  $\approx$  10 gm. of the original liver; solids in 1 cc. = 40 mg.; active. No graying among the rats observed.

*Preparation 37A*—A concentrate (from Lilly's liver, equivalent to 4000 gm. of fresh liver) containing 12 gm. of solids was prepared by adsorption on norit A and subsequent extraction for 100 hours with ether from acid solution in a continuous extractor. This ether extract was dissolved in about 50 cc. of water and neutralized with NaOH. An equivalent amount (4.4 gm.) of copper sulfate

was dissolved in about 15 cc. of water and added. The copper salts were filtered and dried in a desiccator. The dried salts were extracted with anhydrous methanol and this extract freed of solvent. The residue was dissolved in  $H_2O$  and freed from Cu by its precipitation with  $H_2S$ . The filtrate was distilled to dryness at reduced pressure. Total solids, 1.65 gm., which were dissolved in 210 cc. of  $H_2O$ . 1 cc.  $\approx$  20 gm. of liver; solids in 1 cc. = 8 mg.; inactive. Graying noted in two out of three rats.

*Preparation 37B*—The methyl alcohol-insoluble salts in the above procedure were dissolved in  $H_2O$ . The filtrate was freed from Cu and solvent and the total solids of 2.1 gm. dissolved in 210 cc. of  $H_2O$ . 1 cc.  $\approx$  20 gm. of liver; solids in 1 cc. = 10 mg.; inactive. Graying noted in two out of two rats.

*Preparation 37C*—The filtrate after precipitation of the Cu salts was dried and dissolved in about 50 cc. of methanol, and acetone was added until no further precipitation occurred. The wax-like material that separated was filtered off and the filtrate freed of solvent. This was dissolved in about 250 cc. of  $H_2O$  and freed of Cu with  $H_2S$ . The filtrate was freed of solvent, leaving total solids of 6.0 gm., which were dissolved in 400 cc. of  $H_2O$ . 1 cc.  $\approx$  10 gm. of liver; solids in 1 cc. = 15 mg.; active. Graying noted in one out of five rats.

# DISCUSSION

The attempts to concentrate the rat filtrate factor (or factors) have resulted in a separation of two fractions, one extractable from acid aqueous solution with diethyl ether and the other non-extractable. Woolley *et al.* (22) have used this method in the concentration of the "chick antidermatitis factor." This separation is in agreement with the findings of Hoffer and Reichstein (2), El Sadr and coworkers (8), and Subbarow and Hitchings (9, 10). Hoffer and Reichstein and Subbarow and Hitchings have evidence indicating that pantothenic acid (chick antidermatitis factor) is the active component of their ether extracts. We have prepared an isoamyl alcohol extract (a method used by Elvehjem and Koehn (23, 24) and Lepkovsky and Jukes (25) in preparations of chick antidermatitis factor, and by El Sadr and coworkers and Subbarow and Hitchings in preparation of the rat filtrate factor). This preparation after being heated with 1 N NaOH for 1 hour at 100°

retained more than one-half of the original activity. This treatment was shown by Woolley *et al.* (11, 12) to destroy the chick antidermatitis factor.

Our preparations were adsorbable on norit, as reported by Edgar and Macrae (4). Our preparations distilled in a high vacuum, although Edgar and Macrae reported that the activity remained in the residue. Our distillation, however, was carried out at 100° and 10<sup>-5</sup> mm. of Hg pressure, a lower temperature and pressure than were employed by the English workers. Our preparations were likewise soluble in ethanol, methanol, and acetone.

The activity was not destroyed by acetylation. No precipitate formed with either semicarbazide hydrochloride or benzoyl chloride and, although a crystalline material separated on standing with benzylthiuronium chloride, the activity remained in the filtrate. The filtrate from this preparation (No. 29C) was the most active fraction tested (3 mg. of total solids six times weekly resulted in a gain in weight of 56 gm. above the controls). The filtrate factor (or factors) were not precipitated as their Cu salts when treated with CuSO<sub>4</sub>.

In agreement with Morgan, Cook, and Davison (18) and Lunde and Kringstad (19) we have noted frequently but not invariably a graying of the pelage in rats on diets deficient in filtrate factor. In a total of 78 solidly colored or hooded rats serving as controls, 59 showed graying to some extent. No definite areas were regularly affected. In some cases graying was noted in a circular area round the base of the tail and on the forehead; often in the latter area, the graying surrounded a diamond-shaped area of natural color. In other cases the central area of the back was grayed, with a natural colored area surrounding the base of the tail. With some animals the sides were grayed and the central area was of natural color. In a few instances the entire pigmented areas were grayed.

Preparations active in growth stimulation in some cases did not contain the "antigraying factor." As mentioned in a preliminary publication (26), the antigraying as well as the growth factor appears to be extractable with ether, although the growth factor not possessing the antigraying properties remains in the residue.

Lunde and Kringstad (27) and Oleson, Elvehjem, and Hart (28) were unable to correlate graying with growth.

Brewers' yeast (Table I), an excellent source of the growth factor, appeared to be a poor source of the antigraying factor. This observation, no doubt, can explain the not infrequent "stippled" fur observed in animals on purified diets containing yeast as a source of the vitamin B complex.

Cane molasses, the aqueous rice bran extract (Galen B), and liver (Lilly, 92 per cent alcohol extract) are good sources of both factors.

Dried whole milk powder was a poor source of both factors.

#### SUMMARY

Procedures are described for the concentration of the rat filtrate factor complex. The complex is extractable with isoamyl alcohol and is soluble in methanol, ethanol, and acetone. The complex is adsorbable on norit from acid solution. It is not destroyed by heating with 1 N NaOH for 1 hour. It is not inactivated by acetylation. The complex was not precipitated by semicarbazide hydrochloride or benzoyl chloride nor was a benzylthiuronium salt or a Cu salt formed. Two factors at least appear to be a part of the filtrate complex; one is essential for normal growth, the other preserves normal color in the pelage. While both factors are extractable with diethyl ether from acid solutions, the residue, while promoting growth, does not contain the antigraying factor.

Cane molasses, an aqueous rice bran extract, and an alcohol-soluble liver preparation appear to be good sources of both factors but brewers' yeast, while a rich source of the growth factor, is low in the antigraying factor; whole milk powder is low in its content of both factors.

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## ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY

### VIII. THE COMPOSITION OF THE SURFACE FILM ON THE FAT DROPLETS IN CREAM

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The protein composing the stabilizing film on the fat droplets in cream has been identified by various investigators as albumin (1), a glycoprotein (2), a globulin-like protein (3), a new milk protein (4), and casein (5). Some of these results undoubtedly were complicated by an incomplete removal of other proteins in the milk plasma from those naturally occurring on the surface of the fat globules in milk.

To insure a more complete isolation of the materials composing the surface of these globules, Palmer and his associates (6-10) have used the procedure introduced by Storch (2) of washing cream by repeated centrifugation and resuspension in distilled water. They find (9) that the composition of the fat globule surface is not constant in various samples of cream but becomes constant for any sample after four such washings and remains so, at least until the eleventh washing. They conclude that, although milk may contain other stabilizers aside from those at the surface of the fat droplets, the fat globule "membrane" is composed of a tightly adsorbed mixture of phospholipids and a protein differing in constitution (7, 8), behavior (6, 7, 9, 10), and serological reactions (11) from other known proteins in milk. This "membrane" protein was not found in skim milk but could be isolated from buttermilk. The pH of maximum precipitation of both the "membrane" protein and the washed fat droplets was found to lie between pH 3.9 and 4.0 (7). This value is more acid than the isoelectric point of any other protein constituent of milk so far investigated. Palmer and his coworkers (3, 7, 9) have further emphasized the importance of the rôle played by

the phospholipid fraction in the membrane complex. Their value for the pH of maximal precipitation is not to be regarded as invariant, in view of the natural variation in the constitution of this complex.

It would be desirable to employ here the technique of electrophoresis in a direct investigation of the electrokinetic behavior of the membrane complex and to compare the results with the available data for the behavior of other known milk plasma constituents. In other fields, at least, this method has met with success in the interpretation of surface phenomena (12-14). Unfortunately, in the available literature on the electrokinetic properties of the fat droplets, such comparisons appear to be lacking.

Mommsen (15) has found that milk, when suspended in mixtures of 0.2 M NaOH and 0.2 M acetic acid, exhibited an isoelectric point at pH 4.1 in a simple microelectrophoresis instrument constructed from a microscope slide. He found that the behavior of his suspensions changed with time after the electric field was turned on, so that polarization or some other disturbing factor may have been present. Prieger (16), using a similar cell, found that the fat droplets from milk suspended in these buffers were isoelectric at pH 4.2, while butter fat, simply emulsified in distilled water without an added stabilizer, was isoelectric at pH 2.8. On the other hand, a 1 per cent emulsion of butter gave a value of pH 3.95. Mohr and Brockmann (17), using undiluted raw milk, found an isoelectric point at pH 4.3. The ionic strength was a little higher than that of milk itself, owing to addition of concentrated lactic acid to obtain this pH value. The lack of non-polarizable electrodes in some of these earlier investigations, together with irregularities met with in the use of exceedingly shallow cells (12) (some were only 0.1 mm. deep), has led later workers to more modern techniques.

North and Sommer (18) have investigated the streaming potentials produced by adsorbed films prepared by exposure of a butter fat surface, composing the wall of a pore, to dilute skim milk. After treatment of the fat with this milk, the excess was washed away and electrolytes forced through the pore. The assumption is made that (1) this treatment is adequate to produce complete coating and (2) these films are so tightly held that they are not altered by further washing. That such assumptions are

not always valid for other proteins, at least at high dilutions, has been indicated in other papers (19, 20). They present measurements at various pH values, pH 4.3 being obtained as the isoelectric point. This is an interpolation between values at pH 4.12 and 4.68. Measurements are presented on the effects of salts on the streaming potential. The influence of the ionic strength on the isoelectric point (12, 13, 20) does not seem to have been considered. Measurements of streaming potentials involve the determination of the specific electrical conductance of the electrolyte in the pore, thus correcting for possible effects of surface conductance (12, 21). These conductance measurements are, however, about 10 times smaller than corresponding values for KCl in the literature (summarized by Gorin (22)). This is surprising, for, if surface conductance were coming into play, the values would have been expected to be higher, rather than lower. In any case, Rimpila and Palmer have pointed out that skim milk does not contain appreciable quantities of the "membrane" protein, so that this skim milk-butter fat interface can hardly be comparable to the normal fat droplet surface (9).

Seifriz (23) has determined the pH-mobility curve of milk fat droplets suspended in buffer solutions and measured in a micro-electrophoresis instrument. Only relative mobilities are given. The fat droplets were isoelectric at pH 4.55 but the ionic strength was not stated. Nugent (24) has found a value of pH 4.1 for this point in 0.1 M buffers. An isoelectric point at pH 4.3 has been found by Jack and Dahle (25) for fat globules diluted 1:200 with buffer. They present an electric mobility-pH curve but it is not clear whether this was measured in solutions at a total molarity or an ionic strength of 0.25. They conclude that the electrophoretic behavior of the normal milk fat droplets and of butter fat emulsified with casein agrees closely.

In most cases, the results obtained were compared with the isoelectric point for casein, pH 4.6, found by Michaelis and Pechstein (26), without regard to (1) the shift produced in the isoelectric point of casein by different ionic species found by Michaelis and Szent-Györgyi (27), (2) the effect of the ionic strength on the position of the isoelectric point, and (3) possible differences in the isoelectric points of different preparations of this poly-disperse protein.



## 32 Surface Film on Fat Droplets in Cream

In this communication, a direct comparison will be made under comparable conditions of the mobility of washed and unwashed fat droplets with the other proteins in milk for which modern data are available.

### *Methods*

Fresh unpasteurized cream was centrifuged and the underlying liquid removed. Distilled water was then added to the original volume and, after stirring, the process was repeated for six washings. Several drops of this "washed cream" were then added to 50 cc. of buffer solution for the electrophoresis measurements. "Unwashed" suspensions were prepared by adding a similar amount of unwashed cream to the same volume of buffer. All buffers were at a constant ionic strength of 0.02. The casein had been prepared by the method of Van Slyke and Baker (28) and was the same sample as that used by Moyer and Moyer (29). In the preparation of casein sols the methods were the same as described before (29).

Electrophoresis and pH measurements were made according to our usual procedures (30). A flat, horizontal Abramson micro-electrophoresis cell which had been repeatedly checked against other instruments was used. Special care was taken only to measure droplets exactly in focus at the stationary levels. Use of a 40 $\times$  water immersion objective with a 1.9 mm. working distance and a 28 $\times$  ocular (30) made it possible to restrict the focus to a very thin plane, so that all particles in sharp focus would be in the same electroosmotic level.

### EXPERIMENTAL

The data of Moyer and Moyer (29) for the electric mobility of casein are shown in Fig. 1. Inasmuch as casein is highly soluble at pH values above 5.8 at the concentrations we employed, the data above this value were obtained by observing the mobility of quartz or collodion particles coated with films of adsorbed casein. Below this pH value, it is unnecessary to use microscopic particles, for casein itself becomes decreasingly soluble as its isoelectric point is approached, forming microscopic suspensions.<sup>1</sup> It will

<sup>1</sup> Possible occlusion of air at the surface makes it unsafe to make measurements on particles of ground-up, rather than precipitated, casein.

be seen in Fig. 1 that these two sets of data apparently merge into each other, indicating that there is no significant difference in the behavior of the adsorbed films and the precipitated casein particles.

In Fig. 1 are also shown the electric mobilities of unwashed milk fat globules at various pH values. The results above pH 6.5 were secured by suspending the droplets in phosphate buffer mixtures, whereas below this value the globules were measured in sodium

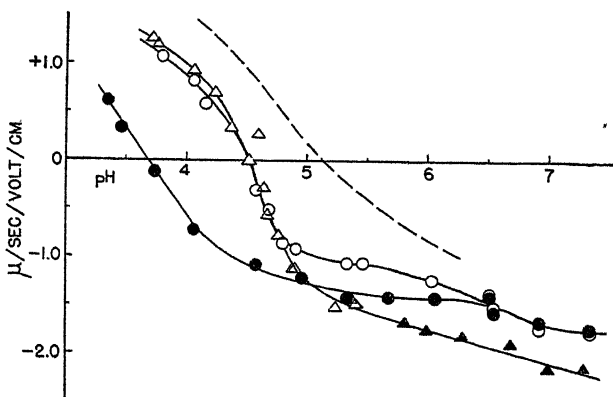


FIG. 1. The electric mobility-pH curves of fat droplets from washed (●) and unwashed (○) cream compared with the behavior of other milk proteins at the same ionic strength,  $0.02 \mu$ .  $T = 25^\circ$ .  $\triangle$  casein particles;  $\blacktriangle$  casein adsorbed on quartz or collodion; dash curve, data of Pedersen for the lactoglobulin of Palmer.

acetate-acetic acid solutions at the same constant ionic strength. The isoelectric point of these globules lay near pH 4.5.

In Fig. 1 is also shown the behavior of fat droplets washed six times with distilled water before suspension in the buffer. It was found that less washing tended to give erratic results, with values lying intermediate between these data and the results for unwashed globules. These droplets had an isoelectric point at pH 3.7. It was noticed at this pH value that complete immobility was not secured; some of the droplets migrated slowly to one, and others to the opposite pole. On Fig. 1 is shown the curve for the electric mobility of the lactoglobulin of Palmer (31), plotted from the results of Pedersen (32) obtained with the moving boundary method.

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Fig. 2 shows the effect of ionic strength on the isoelectric point of casein measured in sodium acetate-acetic acid solutions. These values were obtained by finding, in each case, the pH at which the casein particles did not move. The present, more accurate value for the isoelectric point of casein at  $0.02 \mu$  has necessitated a slight revision of the curve drawn through the measurements of Moyer and Moyer (29) in the neighborhood of the isoelectric point, but this shift only involves a change of about 0.06 pH unit. It will be noticed in Fig. 2 that the isoelectric point is decreased by ionic strengths above 0.02.

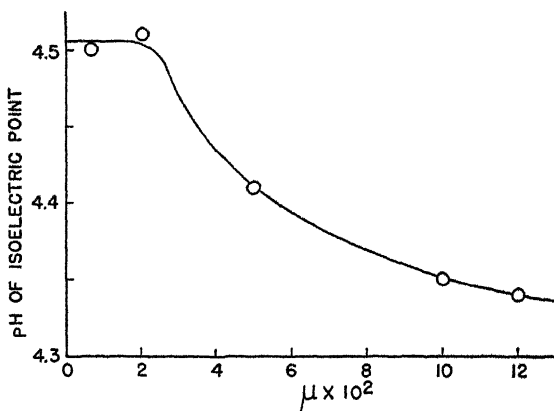


FIG. 2. The influence of the ionic strength,  $\mu$ , of sodium acetate-acetic acid buffers on the pH of the isoelectric point of casein particles.

### DISCUSSION

It is clear from Fig. 1 that the washed fat droplets are completely different in their electrokinetic behavior from the unwashed droplets. The isoelectric point of the washed droplets is in good agreement with the results of Palmer for the pH of maximum precipitation of his "membrane" protein and washed fat droplets. It also agrees with the results of Prieger for the isoelectric point of butter (pH 3.95). Our value for the isoelectric point is not looked upon as an entirely constant quantity in this instance, for it should be affected by changes in the ratio of phospholipids to proteins composing the surface film. This ratio, as Rimpila and Palmer have pointed out (9), is not com-

pletely constant from sample to sample. Additional measurements of the isoelectric point of fat droplets from another sample of washed cream yielded a value of pH 3.8. These results appear to substantiate the conclusions of Palmer and his associates that the washed fat droplet of milk is covered by a film different from the other constituents in the milk plasma and capable of preventing coalescence of the fat droplets even after numerous washings. It is evident that these films are quite different from casein or the lactoglobulin of A. H. Palmer. That they are different from the other proteins which have been suggested as forming their surface has already been demonstrated by Wiese and Palmer (7, 8) and by Palmer and Lewis (11).

On first glance, it may be thought that our data for unwashed cream (Fig. 1) indicate that these droplets possess an outer coating of casein, for, below pH 4.8, the curves for these droplets and for casein follow the same course, within the limits of error. Closer inspection reveals, however, that above pH 6.5 the surfaces of unwashed and washed droplets suspended in buffers at this dilution are not significantly different in their behavior. It is clear that the washed cream does not contain casein on the surfaces of its globules, so that this agreement with the unwashed cream at these higher pH values suggests that these droplets too are not coated by an outer layer of casein. This evidence is strengthened by the fact that these data in this portion of the curve do not agree with the results for casein. It is only below the pH value of fresh milk, approximately pH 6.6, that the results for washed and unwashed fat globules begin to diverge. Below pH 6.0, solutions of casein begin to assume an increasing opalescence with increase of acidity. Hence it seems altogether probable that casein, brought into the unwashed suspensions along with the fat droplets, is becoming increasingly insoluble and accumulating on the surfaces of the fat droplets which are normally unaffected by dissolved casein. As the solubility decreases, below pH 5.0, the outer coating would become more completely casein. It is possible that the fat droplets act as nuclei for the precipitation of casein or it may be that casein is only able to interact with the film on the droplets over a limited pH range. The isoelectric point for these unwashed droplets, while agreeing with the results of Seifriz (23), is only in fair agreement with other

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data in the literature. If these measurements had been made at a different ionic strength or with a different buffer, the peptizing conditions might well have been different, producing isoelectric points intermediate between these and the results for thoroughly washed droplets. The measurements do not tell us about the nature of the outer surface of the fat droplets in undiluted, unwashed cream but they do indicate that, whatever the outer coating may be, the membrane complex of Palmer apparently becomes exposed upon simple dilution, provided that the pH is held between pH 6.0 and 7.5. If the pH is to be lowered below this range, washing is necessary to prevent complications at the surface introduced by the adsorption of casein and the nature of the membrane complex may be hidden.<sup>2</sup>

Fig. 2 shows that it is unsafe to compare isoelectric points of milk fat droplets or other particles measured at one ionic strength with the isoelectric point of casein at another ionic strength. The results of Michaelis and Pechstein (26) for the isoelectric point of casein in the absence of added salts, pH 4.6, are not in complete agreement with these measurements with Van Slyke and Baker casein, but it will be noticed that the agreement between the isoelectric point of the unwashed fat droplets and that for the present sample of casein is excellent, suggesting that this protein has not been changed very much in its electrokinetic properties by purification.

### SUMMARY

An investigation of the surface properties of the fat droplets of cream by means of the microscopic method of electrophoresis has led to the following conclusions.

1. Milk fat droplets washed with distilled water and suspended in buffer solutions exhibit electric mobilities which are markedly different from those of casein under the same conditions, with the isoelectric point considerably lower.

<sup>2</sup> Jack and Dahle (25) report that the mobility of fat droplets washed with distilled water does not become constant but increases as washing proceeds. Their measurements appear to have been made in distilled water, so that a large part of this increased mobility could be due to an increase in double layer thickness with decrease in ionic strength (12). The use of distilled water in electrophoretic measurements of this character has already been criticized (30).

2. Although unwashed fat globules were not significantly different in their behavior from washed fat droplets at values above pH 5.8, below this figure the electric mobility curves were markedly different, with the unwashed droplets progressively assuming an electrokinetic behavior more nearly identical to that of casein as the pH was decreased. The isoelectric point of the unwashed droplets was not significantly different from that of casein.

3. It is suggested that the behavior of the unwashed droplets is complicated by the presence of casein and that at pH values at which casein is very slightly soluble the fat droplet surface becomes contaminated with casein.

4. The isoelectric point of casein is dependent to a certain extent upon the ionic strength in acetate buffers, becoming lower in pH value at higher ionic strengths.

5. These data appear to be most closely in accord with the evidence of L. S. Palmer for the existence of a complex of phospholipids and a "membrane" protein, different from other known milk proteins, composing the fat droplet surface.

It is a pleasure to thank Dr. M. H. Gorin and Dr. L. S. Palmer for their critical reading of the manuscript.

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# VISCOSITY OF SOLUTIONS OF DENATURED AND OF NATIVE EGG ALBUMIN

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There have been several equations derived which express the relation between the viscosity of a suspension and the degree of asymmetry of the particles. Among these equations may be mentioned that of Kuhn (7) which has been employed by Lauffer (8), Neurath and Frampton (10), Neurath and Saum (11), and Neurath (9) to estimate the ratio of the length to the diameter of the protein molecule. These studies have been most interesting and it is largely due to their stimulation that the present research was done.

Recently, Polson (13) has formulated an empirical equation relating the asymmetry of protein molecules to the viscosity of their solutions. At the present stage of our knowledge of the complicated relation between asymmetry and viscosity, an empirical equation such as Polson's seems preferable to one derived on a theoretical basis involving more arbitrary assumptions.

A search of the literature reveals that the viscosity of solutions of native, of heat-denatured, and of urca-denatured egg albumin has been determined by various investigators. The data reported are, however, not suitable for the present calculations, since they were in general determined at higher protein concentrations at which the viscosity to protein concentration relation departs from a linear course. This point is particularly important in the case of the denatured protein. Some of the measurements have also been made without regard to the electroviscous effect and there has been a curious absence of description of the apparatus and experimental conditions under which the viscosity measurements were obtained. The careful and exact technique of Jones and



coworkers on the viscosity of electrolyte solutions demonstrates that the determination of viscosity by the use of the Ostwald viscosimeter presents many difficulties which must be resolved and it seems evident that the experimental conditions for viscosity measurements of protein solutions should be rigorously defined and described.

#### EXPERIMENTAL

Egg albumin was prepared from fresh hen's eggs by the method of Kekwick and Cannan (6). The albumin was recrystallized four times and dialyzed against running distilled water until sulfate-free. The various preparations were between 97.5 and 99.0 per cent heat-denaturable. All preparations were water-clear.

The viscosimeter was of the Ostwald type and made of Pyrex glass. The drainage bulb was cylindrical in shape over nearly its whole length. The reservoir "bulb" was a cylinder of uniform diameter over its entire length with a 45° cone connecting it to the transverse tube which led to the capillary.

The viscosimeter had the following dimensions and characteristics: diameter of reservoir tube (inside) 1.65 cm., diameter of drainage bulb (inside) 1.00 cm., diameter of capillary (estimated from rate of flow) 0.0592 cm., length of capillary 20.1 cm., volume of drainage bulb 3.91 cc., usual working volume 10.0 cc., time of outflow of water at 35° (with 10 cc.) 98.75 seconds, average pressure head (with 10 cc.)  $1.92 \times 10^4$  dynes per sq.cm.

The viscosimeter was clamped in a rigid metal frame and never removed from it. Dichromate cleaning solution was allowed to stand in it when it was not in use. Before a measurement it was washed repeatedly with water and dried by sucking air through it. After a series of readings were made on a solution, it was washed out with hot  $\text{Na}_3\text{PO}_4$  solution and then with water and dried. This treatment was found to restore the water constant. The water constant was determined each day and was found in the course of 9 months to have dropped gradually from 99.07 seconds at the beginning to 98.75 seconds at the end. The solutions were placed in the viscosimeter with a standardized pipette.

The experiments were carried out at  $35^{\circ} \pm 0.01^{\circ}$  in a well stirred water bath. It was found to be most important to have adequate stirring. The time of outflow was determined with a 10 second dial stop-watch and the time could be estimated to 0.01 second. The accuracy of timing was greatly improved by the following technique. Two 0.5 cm. lengths of rubber tubing were placed around the glass tube above the drainage bulb, as well as around the capillary below the drainage bulb, in such a fashion as to expose the calibration marks as narrow slits. A strong beam of light was allowed to strike the viscosimeter at right angles to the observer. Under these conditions the glass tubes at the calibration marks when filled with liquid reflected no light, but when empty appeared brilliantly illuminated. It was frequently possible under these circumstances to achieve a series of outflow timings which did not differ more than 0.01 second from one another.

It was found important never to allow the protein solution to dry in the capillary between measurements, but to draw the solution back into the drainage bulb immediately after an observation had been made; otherwise air bubbles and bits of coagulated protein would cling to the capillary. If the solution was drawn back into the drainage bulb immediately after a measurement, the small amount of surface-coagulated protein which always formed would be carried into the tube above the drainage bulb and would deposit there as the meniscus dropped on a subsequent measurement. In spite of these precautions, however, air bubbles and threads of denatured protein occasionally formed in the capillary and these were removed with a drawn-out glass rod manipulated through the upper tube of the drainage bulb. The tendency to form air bubbles was greatly decreased by boiling the distilled water which was used to make up the diluted protein solutions.

It was established that the viscosity of protein solutions as measured by this viscosimeter was independent of rate of flow, by using working volumes of 5, 10, 15, 20, and 25 cc. Since the reservoir tube was a cylinder of uniform diameter, increasing the working volume by 5 cc. amounted to decreasing the average pressure head by 2.34 cm. of water. The constancy of the viscosity of a 0.2 per cent solution of heat-denatured protein at pH

9.44 with varying working volume is shown by the following figures.

Working volume cc.	$t/t_0$
5.0	1.0237
10.0	1.0248
15.0	1.0236
20.0	1.0236
25.0	1.0234

Since the viscosity is independent of rate of flow, it was considered unnecessary to apply a kinetic correction, as a kinetic correction should vary with rate of flow. The figures above also indicate the absence of structural viscosity. This experiment was repeated at pH 4.19 and at pH 2.50 and both showed constant viscosity with varying rate of flow.

The specific viscosity (specific viscosity is equal to the relative viscosity minus 1) was studied as a function of protein concentration. To our amazement the specific viscosity to protein concentration curve showed a hiatus. It was found that when actual times of outflow were plotted against protein concentration the curve when extrapolated to zero concentration intersected the time axis at a point greater than the water constant of the viscosimeter, in spite of corrections having been made for the viscosity of electrolytes present (see Fig. 1). The difference between the time of outflow of the electrolyte solution and that found by extrapolation of the concentration-time curve, varied with pH, being, in general, lower in the basic region than in the acid and disappearing in the extreme acid and basic regions. At pH 2.60 the difference in times of outflow was 0.50 second and at the isoelectric point it was 0.22 second. Away from the isoelectric point the hiatus in the specific viscosity-concentration curve did not come until concentrations higher than 0.3 per cent of protein had been reached and, in many cases, especially on the acid side of the isoelectric point, the hiatus was not realized even at 0.8 per cent, which was the highest protein concentration used. The extrapolated time of outflow of both heat- and urea-denatured protein solutions was always normal and equal to that of the water constant of the viscosimeter when corrected for the presence of electrolytes.

Much time and effort were spent to ascertain the cause of the extraordinary hiatus in the viscosity to protein concentration relation. To this end the following experiment was done. The clean viscosimeter was filled with protein solution and allowed to stand for a few minutes with the solution in it. The solution was then withdrawn and the instrument washed thoroughly with water and finally dried. It was found that after this treatment the water constant had been increased by 1.00 second. The in-

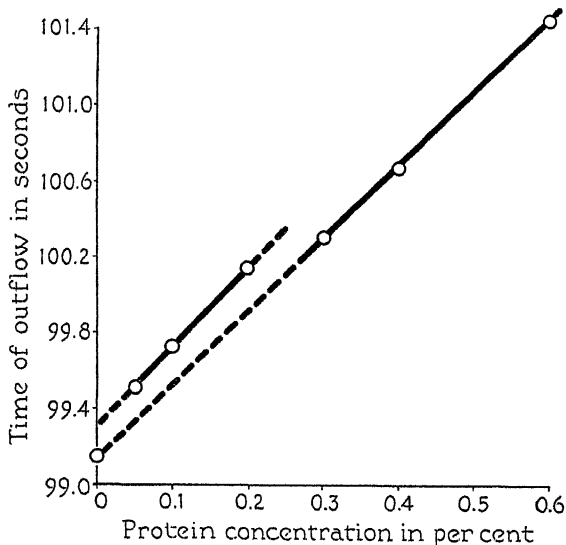


FIG. 1. Time of outflow of egg albumin solutions at the isoelectric point as a function of protein concentration.

strument was then thoroughly cleaned with  $\text{Na}_3\text{PO}_4$  solution, washed with water, and dried. Protein solution was then placed in the reservoir tube but not allowed to enter the capillary. After standing, the protein solution was withdrawn and the instrument washed with water and dried. The water constant under these conditions was 0.92 second above that of the clean instrument. This indicated that conditions produced by the presence of protein in the capillary had little if anything to do with the hiatus in the viscosity with increasing concentration. It was noticed that after the inside of the viscosimeter had been coated with protein by

allowing a protein solution to stand in it, which was then washed out, the water draining from the drainage bulb made a 0° angle of contact with the glass surface, while the ascending meniscus in the reservoir tube made a 90° contact angle. With protein solutions the descending meniscus always made a contact angle of 0° with the surface of the drainage bulb (surface completely wet). The ascending meniscus in the reservoir tube made angles of contact less than 90° but only with higher protein concentrations was complete wetting achieved. This behavior suggested an explanation based upon surface tension effects.

Jones and Fornwalt (5) have considered the corrections which are necessary when one is dealing with a liquid whose surface tension is lower than that of water. Here the situation is somewhat different. The surface in the drainage bulb is being continually destroyed and created as the protein surface is deposited on the interior of the drainage bulb as the meniscus descends. Under these circumstances the surface tension must be high and fairly close to that of water. In the reservoir tube, however, the surface is not destroyed and upon aging surface tension is probably in the neighborhood of 46 dynes per cm. (unpublished experiments by the author on the surface tension of egg albumin solutions). In addition, as has already been pointed out, the ascending reservoir meniscus does not completely wet the glass surface, so that the surface tension is not completely effective in tending to draw the liquid up in the reservoir tube.

The following simple calculation has been carried out for the case of pure water in the viscosimeter which had been previously coated with protein and whose ascending meniscus in the reservoir tube made a 90° angle of contact with the glass surface; from Poiseuille's law of liquid flow in a capillary we have

$$V_1 = \frac{\pi a^4 t_1 P}{8S\eta}$$

where  $V_1$  is the volume of liquid flowing in time  $t_1$ ,  $a$  is the radius of the capillary,  $S$  is the length of the capillary,  $\eta$  is the viscosity of the liquid and  $P$  is the pressure forcing the liquid through the capillary.  $P$ , the average pressure head, was calculated in the following manner. The time of outflow of pure water in the clean viscosimeter with a 10 cc. working volume was determined. The

working volume was then increased by 5 cc. and the new time of outflow obtained. The addition of 5 cc. of water was equivalent to decreasing the average pressure head by 2.34 cm. of water. Two simultaneous Poiseuille equations were obtained. By eliminating the constants common to the two equations, an expression was obtained for the average pressure head in terms of the two times of outflow and the decreased pressure head due to the addition of the 5 cc. of water. The average pressure for a 10 cc. working volume was calculated to be  $1.92 \times 10^4$  dynes per sq.cm.  $a$ , the average radius of the capillary, was calculated by substituting the average pressure head and the other known quantities in Poiseuille's equation and solving for  $a$ .

For a liquid making a contact angle of  $90^\circ$  with the reservoir tube, however,  $P$  has been decreased by the pull exerted by the surface tension of pure water on the wall of the reservoir tube, so that we have in this case

$$V_2 = \frac{\pi a^4 t_2}{8S\eta} \left( P - \frac{2\sigma}{r} \right)$$

where  $\sigma$  is the surface tension of pure water at  $35^\circ$  and assigned the value of 70.7 dynes per cm. and  $r$  is the radius of the reservoir tube and is equal to 0.825 cm. Equating volume  $V_1$  to volume  $V_2$ , and transposing, we have

$$\frac{t_1}{t_2} = 1 - \frac{2\sigma}{Pr}$$

Substituting numerical values, we find that  $t_1/t_2$  is equal to 0.9919, or, since  $t_1$  is equal to 98.75 seconds,  $t_2$  is equal to 99.65 seconds and the difference between the water constant of the clean viscosimeter and that whose interior surface is coated with adsorbed protein is 0.90 second, which compares favorably with the observed difference of 0.92 second. This calculation shows that surface tension effects are entirely capable of accounting for the increased water constant of the viscosimeter. The hiatus in the viscosity-concentration curve is probably due to abrupt changes in the protein surface film as the protein concentration is increased. Bull (1) has already described such changes taking place at about 0.2 per cent egg albumin concentration. Such changes would be expected to alter the wetting qualities of such a film.

*Results*

Fig. 2 shows the specific viscosity as a function of protein concentration for solutions of native, of heat-denatured, and of urea-denatured egg albumin in the presence of 0.2 M phosphate buffer at pH 8.00. The heat-denatured solutions were made by adjusting the protein solutions to pH 8.00 with 0.1 M NaOH, heating on a boiling water bath for 7 minutes, cooling, and adding the phosphate buffer and making up to volume. The solutions of urea-denatured protein were prepared by adding 1 gm. of urea per cc.

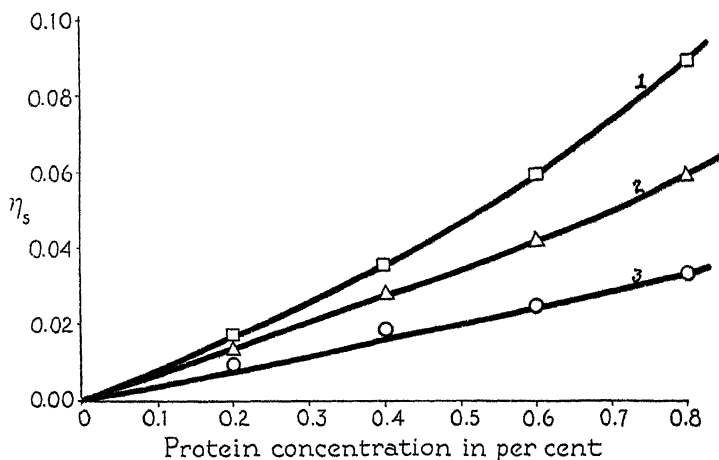


FIG. 2. The specific viscosity of egg albumin solutions as a function of protein concentration. Curve 1, urea-denatured egg albumin; Curve 2, heat-denatured; Curve 3, native egg albumin. All solutions are at pH 8.0 in the presence of 0.02 M phosphate buffer.

of the protein solution. At the end of 1 hour's standing at room temperature the phosphate buffer was added and the solutions made up to volume and their viscosities determined. It will be noted that the viscosities calculated for the 0.2 and 0.4 per cent native protein solutions are slightly above the straight line connecting the values for the 0.6 and 0.8 per cent solutions with the origin (see Fig. 2). This is the same effect as is shown in Fig. 1. The specific viscosity of the heat-denatured and of the urea-denatured solutions did not show this effect and all points for the denatured protein solutions fall on a smooth curve. It is also to

be noted that the viscosities of the solutions of both the urea-denatured and heat-denatured departed from a straight line relation with increasing protein concentration.

The question arose as to what extent the protein in contact with the urea had been denatured. To determine this 1 gm. of urea was added to each cc. of protein solution at the isoelectric point and the solution allowed to stand at room temperature for 1 hour. The solution was diluted and brought back to the isoelectric point with the addition of acid (there is a change of pH upon denaturation), a small amount of  $\text{Na}_2\text{SO}_4$  was added, and the precipitated denatured protein centrifuged and washed twice with water by decantation. The precipitate of denatured protein was finally transferred to a weighing bottle and dried at  $105^\circ$  to a constant weight. That all the urea-denatured protein had been precipitated was established by the fact that the filtrate gave a negative nitroprusside test. This analysis showed that 86.5 per cent of the protein had been denatured by the urea treatment. The viscosities of the solution of urea-denatured protein, as is shown in Fig. 2, really represent, therefore, the viscosity of mixtures of 86.5 per cent urea-denatured and 13.5 per cent native protein. Estimates of the specific viscosity of solutions of completely urea-denatured protein were made by calculating the time of outflow of a native protein solution whose concentration was 13.5 per cent of the total protein concentration. This time of outflow was then used in place of the water constant from which the relative viscosity of the urea-denatured protein solutions, whose concentrations were 86.5 per cent of that of the total protein in solution, were obtained. This calculation assumes the viscosities of the urea-denatured and native protein solutions to be additive.

The volume of protein per cc. of solution was then calculated by multiplying the weight of protein per cc. of solution by 0.746 (specific volume of egg albumin). The values obtained were divided into the corresponding specific viscosities and this function ( $\eta_s/\varphi$ ) plotted against protein concentration. These curves were extrapolated to 0 protein concentration to yield values for  $\eta_s/\varphi$  at 0 protein concentration. The  $\eta_s/\varphi$  values obtained in this manner were 5.5 for native, 9.3 for heat-denatured, and 12.3 for completely urea-denatured protein.



An attempt was made to determine the viscosity of egg albumin denatured by guanidine hydrochloride but it was not found possible to prepare solutions of protein so denatured that were clear and did not contain visibly suspended particles.

#### DISCUSSION

The Polson equation (13) can be written in the following form,

$$\eta_s/\varphi = 4.0 + 0.098 (a/b)^2$$

where  $a/b$  is the ratio of the major and minor axis of a prolate ellipsoid. Substituting the  $\eta_s/\varphi$  values reported above, we have for the native egg albumin molecule an asymmetry of 3.9:1, for heat-denatured 7.4:1, and for urea-denatured 9.2:1, all at pH 8.00 in 0.02 M phosphate buffer.

It is evident from these studies on viscosity that even urea-denatured egg albumin still has considerable structure and is not simply a polypeptide chain in the  $\beta$ -keratin form, since the egg albumin molecule, if it existed as a  $\beta$ -keratin chain, should be about 1000 Å. units long and about 10 Å. wide, which would yield an asymmetry of 100:1.

It is interesting to note that Greenstein (3), in a study of the sulfhydryl groups exposed, upon denaturation found 0.5 per cent sulfhydryl expressed as cysteine for heat-denatured and 1.06 per cent for urea-denatured albumin. The greater sulfhydryl for urea denaturation parallels the greater asymmetry of this form of the molecule.

Williams and Watson (14) reported the molecular weight of urea-denatured egg albumin to be 21,000 as determined by the ultracentrifuge when the diffusion constant of a spherical particle of this size was used in the calculation of the molecular weight. This finding is not substantiated by osmotic pressure measurements of Huang and Wu (4) and by Burk and Greenberg (2). It is probable that the apparent discrepancy between the value obtained by the ultracentrifuge and that by osmotic pressure measurements may be due to the fact that the urea-denatured egg albumin molecule is not spherical but has, as shown by this research, an approximate asymmetry of 9.2:1 as calculated by Polson's equation. Perrin's (12) equation relates the asymmetry of a molecule to the ratio of the real diffusion constant to that of a

molecule having the same mass but spherical in shape. Substituting the asymmetry of the completely urea-denatured molecule as determined by Polson's equation in Perrin's equation, we find that the real diffusion constant of the urea-denatured egg albumin is only 0.66 of that of a spherical molecule of the same weight. Substituting this value in the sedimentation equation and using Williams and Watson's sedimentation constant, we find the molecular weight of urea-denatured egg albumin to be 32,000. This value compares favorably with that reported by Huang and Wu of 32,500 and lends support to the degree of asymmetry as calculated by Polson's equation.

#### SUMMARY

1. The technique is described for the measurement of the viscosity of dilute solutions (less than 1 per cent) of egg albumin.

2. A remarkable hiatus in the concentration to apparent viscosity relation is described. The cause of this hiatus is suggested as being due to lack of wetting of the glass surface by an advancing surface of dilute egg albumin solutions. As the concentration is increased, the wetting characteristics of the surface film abruptly change. The more concentrated solutions wet glass completely.

3. The viscosities of solutions of native, of heat-denatured, and of urea-denatured egg albumin have been measured and the asymmetry of these forms of the egg albumin molecule calculated.

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## STUDIES ON BIOLOGICAL OXIDATIONS

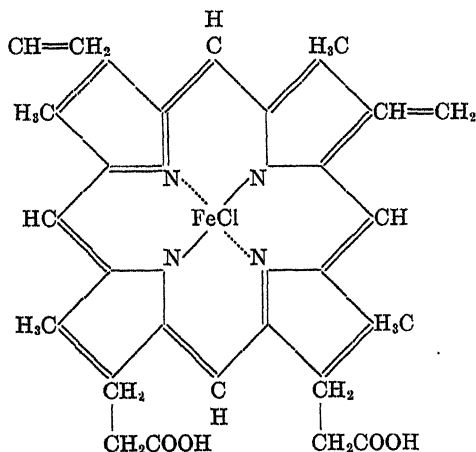
### XIII. THE OXIDATION-REDUCTION POTENTIALS OF SPIROGRAPHIS HEMIN AND ITS HEMOCHROMOGENS

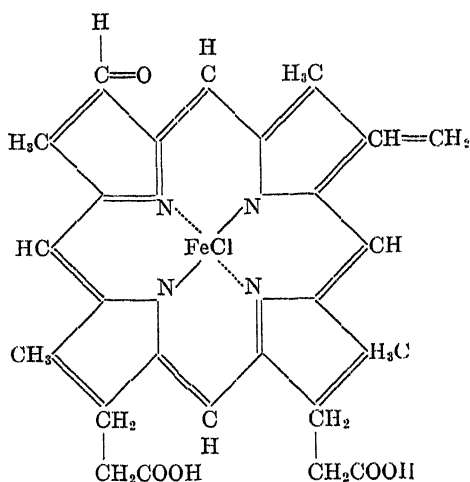
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(Received for publication, December 2, 1939)

Warburg and his coworkers (1, 2) have suggested that the iron porphyrin catalysts which act as the last link for the transfer of electrons from oxidizable substrate to molecular oxygen are of the pheohemin type; they found that the spectrum of the CO compound of *Spirographis* hemoglobin resembled the spectrum of the CO compound of the *Sauerstoffübertragendes Ferment der Atmung*. Warburg *et al.* (3) prepared pure crystalline *Spirographis* hemin, to which the empirical formula of  $C_{32}H_{32}N_4O_5-FeCl \pm 1C \pm 1H$  was assigned. The chemical study of *Spirographis* hemin was continued and brought to a successful conclu-



II. *Spirographis* hemin

sion by Fischer and von Seemann (4). While blood hemin is 1,3,5,8-tetramethyl-2,4-divinylporphin-6,7-dipropionic acid FeCl (Formula I), *Spirographis* hemin is 1,3,5,8-tetramethyl-2-formyl-4-vinylporphin-6,7-dipropionic acid FeCl (Formula II). The replacement of the 2-vinyl group of blood hemin by a formyl group in *Spirographis* hemin is accompanied by a shift of the absorption bands towards the red end of the spectrum (Fox (5), Warburg *et al.* (3)). It has been suggested by Fischer and von Seemann (4) that *Spirographis* hemin is formed by oxidation of a vinyl group of protohemin in position 2. Proceeding with the determination of the oxidation-reduction potentials of iron porphyrins of biological importance, this paper presents data for the potentials of *Spirographis* hemin and some of its hemochromogens.

#### EXPERIMENTAL

The sample of *Spirographis* hemin was kindly furnished in 1932 by Dr. Reid from Professor Warburg's laboratory. Appreciation and thanks for this assistance are here extended to both of them. The titrations were performed with a Rehberg microburette containing 0.2 cc. of titrating solution. The titration vessel was so constructed as to leave the rubber stopper free to receive only the burette tip and two platinum electrodes (Fig. 1).

A mercury seal between the burette tip and the vessel allowed a free vertical motion of the vessel, which was produced by means of a lever (*b*) placed at the bottom of a disk supporting the titra-

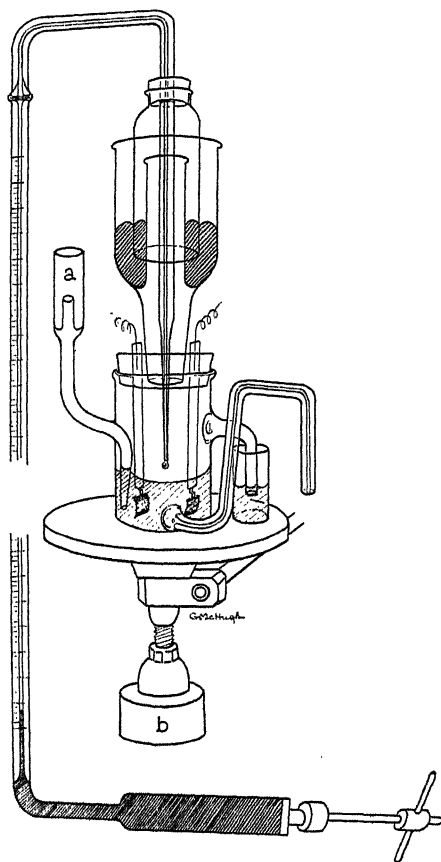


FIG. 1. Apparatus for potentiometric microtitrations. *a* is the opening through which the purified nitrogen is passed; *b*, the lever at the bottom of the disk supporting the titration vessel.

tion vessel. This device makes it possible to get from the burette quantities of reagent as small as 0.001 cc. Purified nitrogen entering the titration vessel through *a* was kept bubbling throughout the titration, and 1 drop of caprylic alcohol was added to the

solution to avoid foam formation. The titration vessel was connected to the calomel electrode by a capillary tube containing agar-saturated KCl which was prepared according to Borsook and Schott (6). The potentials were measured at 30° by a type K Leeds and Northrup potentiometer, with the Michaelis standard acetate (pH 4.62) used as reference electrode. Titrations were performed on 10 cc. of 0.0001 M *Spirographis* hemin, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> being used as reducing agent.

#### *Determination of Potentials*

Titration of *Spirographis* hemin was performed at two pH values, 9.63 and 7.224. The same difficulties as those experienced in the titration of blood hemin (7) were found here, the electrodes giving stable E.M.F. values very slowly at the beginning and erratic values at the end of the titration. At a pH value of 7.224, the *Spirographis* hemin was titrated in Clark's phosphate buffer (8) by dissolving the hemin in NaOH solution and adding afterwards the required amount of the KH<sub>2</sub>PO<sub>4</sub> solution. Up to 85 per cent reduction the titration values agreed satisfactorily with calculated values from the equation

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{(\text{Fe}^{++} \text{ hemin})}{(\text{Fe}^{+++} \text{ hemin})} \quad (1)$$

where  $n = 1$ . Beyond that point there was aggregation and the E.M.F. values became erratic. At pH 9.66 the hemin was dissolved in borate buffer. The  $E'_0$  value at pH 7.22 was  $-0.089$  volt; at pH 9.63,  $-0.230$  volt. The  $-dE'_0/d \text{ pH}$  value seemed to be about 60 millivolts per pH unit as in blood hemin.

In the titration of hemochromogens, since the amount of *Spirographis* hemin was insufficient for titrations at different concentrations of nitrogenous base, the same concentrations of base were used as those required to saturate blood hemin (7). Cyanide, pilocarpine, and  $\alpha$ -picoline hemochromogens were prepared and titrations performed with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> in borate buffer at the following pH values: 9.95 for cyanide hemochromogen, 9.63 for pilocarpine hemochromogen and  $\alpha$ -picoline hemochromogen (Fig. 2). Cyanide and pilocarpine hemochromogenes gave  $E_h$  values closely agreeing with those obtained from Equation 1 where  $n = 1$ .  $\alpha$ -Picoline hemochromogen behaved like pyridine hemochromogen

when titrated at pH 10.8 (7), the  $E_h$  values occupying an intermediate position between those given by the electrode equation with  $n = 1$  and those given when  $n = 2$ . This is probably due to

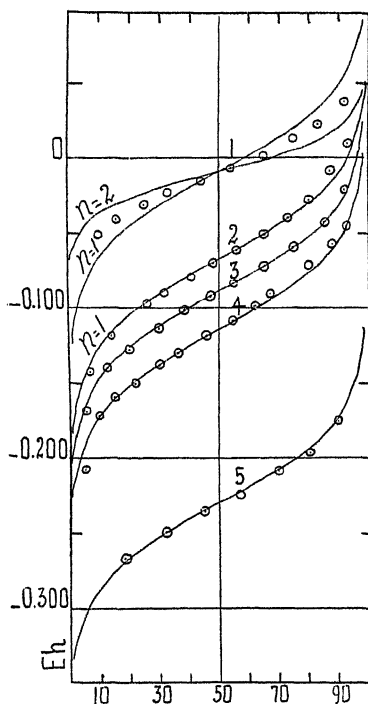


FIG. 2. Oxidation-reduction potentials of *Spirographis* hemin and its hemochromogens. The abscissa represents per cent oxidation; the ordinate,  $E_h$  in volts;  $\bigcirc$ , experimental findings; solid line, theoretical curve for values of  $E_h = E'_0 - (RT/nF) \ln (Fe^{++}/Fe^{+++})$ . Curve 1,  $\alpha$ -picoline hemochromogen titrated at pH 9.63 (concentration of  $\alpha$ -picoline, 1 M); Curve 2, pilocarpine hemochromogen titrated at pH 9.63 (concentration of pilocarpine, 0.06 M); Curve 3, *Spirographis* hemin titrated at pH 7.22; Curve 4, cyanide hemochromogen titrated at pH 9.95 (concentration of KCN, 0.07 M); Curve 5, *Spirographis* hemin titrated at pH 9.63.

polymerization and aggregation. The  $E'_0$  values found by titration of these hemochromogens were as follows:  $-0.113$  volt for cyanide *Spirographis* hemochromogen,  $-0.068$  volt for pilocarpine *Spirographis* hemochromogen,  $-0.010$  volt for  $\alpha$ -picoline



*Spirographis* hemochromogen. In Table I these values are compared with the  $E'_0$  values of blood hemin and its hemochromogens. The oxidation of the vinyl group in position 2 in the porphyrin of blood hemin to give the formyl group in the porphyrin of *Spirographis* hemin is thus accompanied with a shift of the

TABLE I

$E'_0$  Values of *Spirographis* Hemin and Its Hemochromogens As Compared with Those of Blood Hemin and Its Hemochromogens

	pH	$E'_0$ volt
<i>Spirographis</i> hemin.....	9.63	-0.230
Blood hemin.....	9.63	-0.316
<i>Spirographis</i> hemin.....	7.22	-0.089
Blood hemin.....	7.22	-0.128
Cyanide <i>Spirographis</i> hemochromogen.....	9.95	-0.113
“ hemochromogen.....	9.95	-0.183
Pilocarpine <i>Spirographis</i> hemochromogen.....	9.63	-0.067
“ hemochromogen.....	9.63	-0.170
$\alpha$ -Picoline <i>Spirographis</i> hemochromogen.....	9.63	-0.010
“ hemochromogen.....	9.63	-0.033

TABLE II

Position of Maximum Absorption Spectrum Bands of Ferrohemochromogens of *Spirographis* Hemin and Blood Hemin

Hemochromogen	<i>Spirographis</i> hemin	Blood hemin
	$\text{\AA}$ .	$\text{\AA}$ .
Cyanide.....	5675	5365
“ <i>Spirographis</i> .....	5930	5500
Pilocarpine.....	5562	5265
“ <i>Spirographis</i> .....	5883	5441
$\alpha$ -Picoline.....	5555	5215
“ <i>Spirographis</i> .....	5850	5375

potentials towards more positive values. Whether this shift in the potential is accompanied by a change in the catalytic power is not yet known.

The position of the maximum absorption spectrum bands of the ferrohemochromogens here studied is given in Table II, together

with that of the corresponding ferrohemochromogens from blood hemin. In every case the two bands in the visible portion of the spectrum of the *Spirographis* ferrohemochromogens were shifted towards the red end of the spectrum, by 255 Å. in cyanide, by 321 Å. in pilocarpine, and by 295 Å. in  $\alpha$ -picoline hemochromogen. Warburg and Negelein (2) give 584 m $\mu$  for the  $\alpha$  band of pyridine *Spirographis* hemochromogen as compared with 557 m $\mu$  for the hemochromogen of blood hemin. A displacement in the same direction occurs in the great Soret band. Situated in the vicinity of 4000 Å. in blood ferrihemin, it is displaced to 4200 Å. in *Spirographis* ferrihemin (Warburg and Negelein (2)). In cyanide ferrihemochromogen this band has its maximum at 4225 Å. (9); it was shifted to 4500 Å. in cyanide *Spirographis* ferrihemochromogen. The displacement of the absorption spectrum bands of iron porphyrins towards longer wave-lengths when the values of the oxidation-reduction potentials approach that of oxygen, as observed here, will be discussed when the potentials and absorption spectra of pheohemins are reported.

#### SUMMARY

The oxidation-reduction potentials of *Spirographis* hemin (1,3,5,8 - tetramethyl-2-formyl-4-vinylporphin-6,7-dipropionic acid FeCl) and some of its hemochromogens have been determined by electrometric titration at 30°. The  $E'_0$  value of *Spirographis* hemin at a pH value of 7.22 was -0.089 volt; at pH 9.63, -0.230 volt. The  $E'_0$  value of cyanide *Spirographis* hemochromogen at pH 9.95 was -0.113 volt; that of pilocarpine *Spirographis* hemochromogen at pH 9.63 was -0.067 volt; that of  $\alpha$ -picoline *Spirographis* hemochromogen at pH 9.63 was -0.010 volt. In every instance the  $E'_0$  value was more positive than that of blood hemin. The absorption spectrum bands of *Spirographis* hemin and its hemochromogens were shifted towards the red end of the spectrum by about 300 Å. as compared with those of blood hemin.

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## ANTI-KETOGENIC AND GLYCOGENIC ACTIVITY OF CITRIC ACID

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Citric acid has long been known to be readily oxidized by the mammalian organism but until recently the details of its intermediate metabolism have remained obscure. Martius and Knoop (1, 2) have demonstrated that  $\alpha$ -ketoglutaric acid is produced by the action of liver dehydrogenase on citric acid. Krebs and Johnson (3) found both  $\alpha$ -ketoglutaric and succinic acids to be oxidation products of citric acid in isolated muscle tissue and Krebs (4) includes both of these compounds in his "citric acid cycle," a series of reactions through which he believes carbohydrate to be oxidized in animal tissues. Breusch (5) has strongly criticized the evidence for the existence of the citric acid cycle, at least in muscle. We are not concerned here with the details of muscle cell metabolism and it would be surprising if exogenous citric acid could be thrown into such a cycle should it exist. Of more concern here is the nature of the oxidation of citric acid, which may take place in the liver. The findings of Orten and Smith (6) that the injection of all the compounds in the citric acid cycle increased citrate excretion have been taken by Krebs to support his scheme. With his coworkers Krebs (7) confirmed Orten and Smith and found that  $\alpha$ -ketoglutaric acid as well as citric acid was excreted after the injection of the members of the "citric acid cycle." Malonate, believed to inhibit the enzymes concerned with the cycle, caused the excretion of succinic and lesser amounts of  $\alpha$ -ketoglutaric and citric acids. Succinic acid is very definitely a glucose former in the intact mammalian organism. It yields glucose in the phlorhizinized dog (8, 9), forms liver glycogen (10, 11), and is antiketogenic (11). If the route citric acid  $\rightarrow$  . . .  $\rightarrow$   $\alpha$ -ketoglutaric acid  $\rightarrow$  . . .  $\rightarrow$  succinic acid is

correct, citric acid should be glycogenic, a property which is not generally accepted for it. Lusk (12) interpreted an experiment of Baer and Blum (13) as evidence that citric acid could form glucose, as there was an increase in glucose excretion when sodium citrate was administered to a phlorhizinized dog. Greenwald (14) found that the administration of sodium citrate to phlorhizinized dogs and to a patient with diabetes mellitus was followed by an increased excretion of glucose, concluding that there was a conversion of the 6 carbon atoms of citric acid to glucose. Fürth *et al.* (15) have reported that in a phlorhizinized dog citric acid exerted neither an antiketogenic nor a glycosuric effect and failed to find any increase in liver glycogen from feeding it to rats.

The question of the glycogenic action of citric acid has been examined in a number of different ways. The ability (1) to overcome the effects of insulin hypoglycemia, (2) to form liver glycogen, and (3) to exert antiketogenic action has been considered. A study of the ability of citric acid to form glucose in the phlorhizinized dog would also have been desirable and was attempted without success. The marked influence of an alkalosis on citric acid metabolism (16-19) made it necessary to feed the free acid and not the sodium salt. Citric acid solutions, administered by stomach tube, are retained very poorly by normal fasting dogs and the phlorhizinized fasting organism is remarkably sensitive. Experiments were carried out on eleven dogs and in no case was an appreciable amount of the citric acid retained, even when doses of only 2 gm. in 1 per cent solution were used.

*Relief of Insulin Hypoglycemia by Citric Acid*—It is generally agreed that only those substances which, on enzymatic hydrolysis by the tissues or action by the liver, yield glucose are capable of overcoming insulin hypoglycemia. A comparison of the effect of citric acid with glucose in this regard is presented in Table I. Regular insulin of 40 units per cc. strength was used. The glucose and citric acid solutions were administered by stomach tube. Many of the rats which recovered were prostrate or even had convulsions, during the period after the insulin was given, before they recovered.

It is obvious from the data in Table I that citric acid is capable of overcoming insulin hypoglycemia and it would seem probable

TABLE I

*Comparison of Effect of Citric Acid with That of Glucose in Relieving Insulin Hypoglycemia in Rats*

Experiment No.	Group No.	No. of rats	Treatment	Body weight	Average body surface	Result		
						No. died	Time	No. recovered
				gm.	sq.dm.		hrs.	
1	1	3	Controls	154-186	3.5	3	8, 8, 9	0
	2	3	Glucose	159-173	3.4	3	11, 12, 13	0
	3	3	Citric acid	154-179	3.4	0		3
2	1	3	Controls	228-270	4.4	3	14, 15, 17	0
	2	3	Glucose	192-238	4.1	1	18	2
	3	3	Citric acid	232-300	4.6	0		3
3	1	8	Controls	247-345	5.1	5	4, 5, 6, 10, 12	3
	2	8	Citric acid	264-324	5.1	0		8
4	1	3	Controls	177-208	3.9	3	3.3, 3.5, 4.1	0
	2	3	Glucose	177-227	3.9	3	3.5, 3.6, 5.7	0
	3	3	Citric acid	181-215	3.8	3	6.7, 7.3, 11.1	0

*Experiment 1*—Female rats fasted for 48 hours; then given 2.5 units of insulin per sq.dm. of body surface intraperitoneally at the 0 hour of the experiment. The dose was repeated in 5 hours. Solutions of 0.5 M glucose and citric acid were given to the respective groups at 0.5 hour in doses of 1.0 cc. per sq.dm. of body surface. The citric acid was too concentrated and when the doses were repeated at 6.25 hours the citric acid group received only 0.5 cc. per sq.dm. of body surface.

*Experiment 2*—Male rats fasted for 48 hours; then given 5 units of insulin per sq.dm. of body surface subcutaneously at 0 hour. At 3.5 hours all were very weak and were given 0.5 cc. per sq.dm. of the two solutions in concentrations of 0.5 M. At 6 hours these doses were repeated.

*Experiment 3*—Male rats fasted for 72 hours; then given 4 units of insulin per sq.dm. of body surface subcutaneously at 0 hour. Citric acid in 0.25 M solution given to one group in a dose of 1 cc. per sq.dm. of body surface immediately afterward.

*Experiment 4*—Female rats fasted for 24 hours before being given 10 units of insulin per sq.dm. of body surface by the subcutaneous route at the 0 hour of the experiment. At 1.25 hours all were prostrate. At 1.5 hours 0.5 cc. per sq.dm. of body surface of the solutions was given. At 3 hours the rats were all in convulsions and at 3.25 hours the doses of glucose and citric acid were repeated.

from this that it may form glucose in the organism. On a molecular weight basis it would seem to be more active than glucose. We would hesitate to draw any conclusions from this as to the

TABLE II  
*Glycogenic Action of Citric Acid in the Rat*

Ex- per- iment No.	Group No.	No. of rats	Treatment	Body weight			Average body surface sq. dm.	Urine N over entire period per sq. dm.	Liver weight			Liver glycogen			Liv- er glyco- gen per sq. dm.
				Mini- mum	Aver- age	Maxi- mum			Mini- mum	Aver- age	Maxi- mum	Mini- mum	Aver- age	Maxi- mum	
				gm.	gm.	gm.	sq. dm.	mg.	gm.	gm.	gm.	per cent	per cent	per cent	mg.
1	1	6	Controls	137	149	160	3.2	100	1.42			0.18			2.5
	2	6	Citric acid	139	154	184	3.3	94	1.46			2.98			43.4
2	1	12	Controls	234	260	287	4.6	87	1.25	1.43	1.72	0.09	0.09	0.10	1.1
	2	12	Citric acid	239	263	281	4.7	80	1.38	1.58	1.89	1.10	1.28	1.33	20.2
3	1	12	Controls	162	184	261	3.7	30	1.41	1.54	1.62	0.05	0.14	0.17	2.3
	2	12	Citric acid	170	190	224	3.8	32	1.37	1.60	1.86	0.98	1.45	1.68	23.2
4	1	8	Controls	168	214	250	4.1		1.38			0.09	0.27	0.59	3.7
	2	8	Citric acid	170	215	251	4.1		1.35			0.49	0.91	1.48	124.0
	3	8	Sodium bicarbonate	176	217	251	4.1		1.35			0.05	0.07	0.10	1.0
	4	8	" citrate	186	215	246	4.1		1.40			0.10	0.30	0.57	42.0

*Experiment 1*—Male rats fasted for 24 hours preceding the administration of 0.25 M citric acid solution in doses of 1 cc. per sq. dm. of body surface at 0, 9, 17, 21, 33, 41, 56, 59, and 64 hours; killed at 70 hours.

*Experiment 2*—Male rats fasted 48 hours and one group then given doses of 5 cc. of 0.25 M citric acid at 38, 46, 60, 66, 72, and 84 hours; killed at 90 hours.

*Experiment 3*—Female rats fasted for 48 hours and one group then given 4 cc. each of 0.25 M citric acid at 0, 4.5, 7.5, 18, 21, and 23.5 hours; killed at 27 hours.

*Experiment 4*—Male rats fasted 48 hours and then given the following: Group 1, water; Group 2, 0.2 M citric acid; Group 3, 0.6 M sodium bicarbonate; and Group 4, 0.2 M sodium citrate in amounts of 1 cc. per sq. dm. of body surface at 0, 12, 27, and 56 hours; killed at 61 hours.

quantity of glucose which a given amount of citric acid may yield, for as we will show elsewhere the speed of formation of glucose is just as important as the amount of glucose a substance may form in overcoming insulin hypoglycemia. It is probable that citric acid forms glucose rather slowly and for this reason is more active than glucose itself in equimolecular quantities. If we follow Krebs' scheme (4) for the oxidation of citric acid by muscle and assume that the route of glucose formation from citric acid by the liver is the same, 2 moles of citric acid = 1 mole of glucose.

*Formation of Liver Glycogen by Citric Acid*—The glycogenic action of citric acid is quite marked. The experiments in which this is demonstrated are detailed in Table II. The administration of citric acid always caused a definite increase in the amount of hepatic glycogen. Since there was no increase in nitrogen excretion which might be taken as evidence that the extra glycogen arose from protein breakdown, we must assume that the glycogen was formed from the citric acid. This is further evidence that citric acid may form glucose in the organism. It may be seen from Experiment 4 that sodium citrate formed less glycogen than the free acid. This appears to be a result of the influence of an alkalosis on citric acid metabolism, which will be considered elsewhere. The method of Good *et al.* (20) was used for the glycogen determinations.

*Antiketogenic Activity of Citric Acid*—In so far as we are aware any substance which has antiketogenic activity is able to form glucose in the organism and conversely all glycogen formers show antiketogenic action. Since citric acid is a glycogen former, it should be antiketogenic, and this is the case. Following a diet low in protein, a good fasting ketosis ensues in rats (11). The data in Table III were obtained from such fasting rats by methods described elsewhere (11). After removal from the diet the rats were fasted for a day before observations were commenced.

Collection of a blood sample for acetone body determination involved sacrifice of the animal and the number of rats in a group from which urine collections were made was reduced by two whenever blood samples were taken. Urine acetone bodies were determined by Van Slyke's method (21) and blood acetone bodies by the method of Barnes and Wick (22).

The results in Table III show very clearly, both from the urine





excretion of acetone bodies and the blood levels, that citric acid has a marked antiketogenic action. Although the experiments were not designed to make a quantitative comparison of the activity of glucose and citric acid, the data suggest that mole for mole citric acid is about half as active as glucose, which is what theory demands.

#### SUMMARY

In the albino rat citric acid is as effective as glucose in the relief of insulin hypoglycemia, active in the formation of liver glycogen, and has a marked antiketogenic action.

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## THE ESTIMATION OF HISTIDINE

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During our experience with the silver precipitation method for the determination of the basic amino acids, the procedure for histidine has been changed repeatedly. A histidine value of approximately 0.9 per cent for mammalian serum proteins, reported by Block, Darrow, and Cary (1) in 1934 and obtained by precipitation of the di flavianate, was shortly thereafter (2) replaced by the value of 2.1 per cent when the histidine precipitated with silver at pH 7.4 was estimated by a modification of the Kapeller-Adler method. The nitranilic acid method (3), which is now in regular use and yields results agreeing well with the higher values, is here described in detail.

The results given in the experimental part suggest that the histidine present in a protein hydrolysate can be recovered quantitatively by precipitation with silver at pH 7.4 followed by nitranilic acid. It must be remembered, however, that a varying proportion of the histidine may be destroyed during the hydrolysis. Thus Schenk (4), Csonka (5), Block (6), and others have reported the failure to isolate histidine after acid hydrolysis of whole yeast, although no difficulty was encountered by the latter two investigators when the yeast proteins were first separated from the bulk of the carbohydrate impurities.

### EXPERIMENTAL

The experiments described below are only a few of the eighty odd carried out in an effort to improve the determination of histidine in proteins.

Three 135.5 mg. portions of histidine hydrochloride monohydrate (m.p. 255°, N 20.0 per cent, theory 20.0 per cent) were

dissolved in 15 cc. of water and 225 mg. of nitranilic acid dissolved in 25 cc. of water, acetone, or methanol were added. The sides of the flasks were scratched and the solutions were placed in the refrigerator overnight. Histidine nitranilate ( $C_6H_9O_2N_3 \cdot C_6H_2O_8N_2$ ) weighing 249, 251, and 250 mg. was obtained. These values, when calculated as histidine (factor 0.403), indicate a quantitative recovery.

In order to ascertain the recovery of histidine from protein hydrolysates, the following experiments were conducted. 5.000 gm. of gelatin, casein, or cattle blood proteins were hydrolyzed with 50 cc. of 8 N sulfuric acid. The amino acid hydrolysates were diluted to 100 cc. and the solutions were divided in 2 equal parts. 135 mg. of histidine hydrochloride hydrate were added to one portion of each protein hydrolysate and the histidine was isolated as follows: The amino acid solutions were diluted to 200 cc. and warm concentrated barium hydroxide was added to pH 3.5 to 4.0. The barium sulfate was removed by centrifugation and washed twice with hot water. The clear filtrates were concentrated to approximately 100 cc. *in vacuo* and the histidine was precipitated as the silver salt at pH 7.4. The histidine silver was centrifuged off and the precipitate washed with water. The silver salt was then suspended in water, acidified with dilute sulfuric acid, and the silver removed with hydrogen sulfide. The histidine sulfate solution was concentrated to approximately 200 cc. and the pH of the solution adjusted to 3.5 to 4.0 with dilute barium hydroxide. The precipitate was removed and washed. The filtrates were concentrated to 10 cc. and the histidine was precipitated from a 50 per cent methyl alcoholic solution by an excess of solid nitranilic acid. After standing overnight, the histidine nitranilate was filtered on sintered glass crucibles, washed with methanol and ether, and dried at 110°. The following results were obtained.

Gelatin + histidine yielded 266.6 mg. histidine nitranilate\*

“ yielded 21.5 mg. histidine nitranilate\*

Recovery 99%

Casein + histidine yielded 372.8 mg. histidine nitranilate\*

“ yielded 134.0 mg. histidine nitranilate\*

Recovery 96%

Blood proteins + histidine yielded 594.0 mg. histidine nitranilate\*

“ “ yielded 339.6 mg. histidine nitranilate\*

Recovery 103%

\* N = 18.0 to 18.1 per cent; calculated 18.2 per cent.

100 mg. portions of histidine nitranilate (except in the case of gelatin) obtained above were dissolved in a solution of 90 cc. of water, 85 cc. of acetone, 1 cc. of N hydrochloric acid, 1 cc. of 5 N sulfuric acid, and 10 cc. of 25 per cent potassium chloride. The precipitate of dipotassium nitranilate was washed with 2.5 per cent potassium chloride in 50 per cent ethanol. Total nitrogen determinations on the filtrates gave 10.9 to 11.2 mg., calculated for 40.3 mg. of histidine 11.1 mg.

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# **BASIC AMINO ACID CONTENT OF HUMAN SERUM PROTEINS. THE INFLUENCE OF THE INGESTION OF ARGININE ON THE COMPOSITION OF THE SERUM PROTEINS\***

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Dirr (1) has recently reported that the administration of 3 gm. of arginine monohydrochloride intravenously or of 5 gm. orally resulted in a very considerable increase in the arginine content of the serum proteins. In one instance, he reported that the amount of arginine was increased from 5.42 to 8.15 per cent 1 hour after feeding 5 gm. of arginine hydrochloride. Dirr made use of a modification of the Sakaguchi (2) reaction to determine the arginine content of the serum proteins. The importance of this observation prompted us to repeat these experiments.

## **EXPERIMENTAL**

Blood was drawn from one male and one female patient who were recovering from fractures of the leg but who were physically normal in all other respects. The initial sample was taken in the morning before breakfast. The subsequent samples were taken at stated intervals after the administration of 12.5 and 24 gm. of arginine hydrochloride. The blood was allowed to clot and the serum was removed by centrifugation. The sera were acidified with 5 N acetic acid to pH 4.5 and, after the addition of 3 volumes of dilute sodium chloride, the proteins were coagulated by heat. The precipitate was washed three times with hot water, and the lipids were removed by extraction with acetone, hot alcohol, hot benzene, and ether. Nitrogen was determined by the macro-

\* This investigation was aided in part by a grant from Child Neurology Research (Friedsam Foundation).



Kjeldahl method. The basic amino acids were estimated on 2.25 gm. of protein by the modification of the silver precipitation procedure in use in this laboratory (3). Arginine was isolated as the flavianate, histidine as the nitranyl salt, and lysine as the picrate.<sup>1</sup> The purity of the derivatives was controlled by analyses and by melting points.

### Results

The feeding of arginine did not result in any appreciable rise in the arginine content of the serum proteins (Table I). The molecular ratio of histidine to arginine to lysine remained approximately constant in all five experiments. The slight rise in arginine from 3.9 to 4.1 per cent in the male patient is within the experimental error of the method.

In the light of these results, we thought it possible that the increases found by Dirr after the feeding of arginine may have been due in part to the method he used to prepare the serum proteins. The following experiment was, therefore, carried out. 800 mg. of arginine hydrochloride were added to 160 cc. of pooled serum obtained from the above patients. The arginine-containing serum was divided into 2 equal parts and the proteins were isolated from the one portion by precipitation with alcohol, according to Dirr, and from the other portion by heat coagulation. The two

<sup>1</sup> The determination of lysine as described in 1938 has been modified as follows: The lysine sulfate solution before being made alkaline with NaOH is concentrated to approximately 200 cc. rather than 10 to 25 cc. as suggested (*cf.* (3) p. 9). The final steps in the isolation of lysine have also been changed. Thus, the lysine carbonate solution containing barium carbonate in suspension (*cf.* (3) p. 10) is concentrated to approximately 150 cc. and the precipitate is removed by centrifugation. The supernatant liquid and washings are returned to the round bottom flask and the lysine solution is concentrated at low temperature (25–30°) to approximately 25 cc. The solution is filtered through soft paper into a 100 cc. round bottom flask with a ground glass joint and the lysine solution is concentrated further at low temperature to approximately 3 cc. The capillary and sides of the still are washed down with a little water and then thoroughly with absolute alcohol. An excess of picric acid dissolved by warming in absolute alcohol is added to the lysine solution, and the sides of the flask are scratched with a stirring rod on which a little pure lysine picrate has been allowed to crystallize. The solution is permitted to remain in the refrigerator overnight. The lysine picrate which crystallizes out in needles of from 1 to 4 mm. in length explodes sharply around 260° without recrystallization.

protein preparations were analyzed for nitrogen and the basic amino acids. The results of these analyses are given in Table I.

The amount of arginine in the serum proteins precipitated by alcohol, although somewhat higher than that found in the heat-coagulated protein preparation, is hardly greater than that due to the experimental error of the method, and, even if it were significant, it would account for only a small part of the rise reported by Dirr.

TABLE I  
*Basic Amino Acid Content of Human Serum Proteins*

Serum	Time after feeding	Nitrogen	Histidine	Arginine	Lysine
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Male, fed 24 gm. arginine HCl	0	14.8	2.0	3.9	5.9
	1	14.3	2.1	4.1	6.1
Female, fed 12.5 gm. arginine HCl	0	15.2	2.0	4.7	6.8
	1.5	15.2	2.1	4.3	6.2
	3	15.0	2.1	4.4	5.8
Pooled serum + arginine HCl					
Alcohol precipitation		14.9	2.0	4.9	5.9
Heat coagulation		14.9	2.1	4.6	6.0
Control (1939)		15.3	2.0	4.1	5.8
" (1934)		14.7	2.1	4.7	6.9

In order to show that the proteins prepared by precipitation with 2 volumes of alcohol and by heat coagulation were similar in composition to those prepared by precipitation with 5 volumes of acetone, the average results of four recent duplicate analyses of serum proteins prepared by acetone precipitation are given in Table I, "Control (1939)." As a further control, an average of six duplicate analyses carried out in 1933 and 1934 is included in Table I, "Control (1934)." It will be seen that the amino acid composition of the serum proteins prepared by all three methods is approximately the same.

#### SUMMARY AND CONCLUSIONS

1. Human serum proteins prepared by heat coagulation or by precipitation with 2 volumes of alcohol or 5 volumes of acetone have approximately the same basic amino acid composition.

2. Proteins precipitated from serum containing 0.5 per cent of arginine hydrochloride yield little if any extra arginine.

3. In the two human beings studied, the *arginine content of the serum proteins was not appreciably increased by the ingestion of relatively large quantities of arginine hydrochloride.*

I am indebted to Dr. George A. Jervis for the sera and to Merrill Webb for invaluable assistance in the analyses.

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## MINERAL METABOLISM OF RATS ON AN EXTREMELY SODIUM-DEFICIENT DIET\*

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In an earlier paper (14) we have described the symptomatology and effect on reproduction in the rat produced by uncomplicated deficiency of sodium. Little is known of the specific rôle of sodium in mammalian metabolism in its relations to other metabolic products. Since this element has generally been considered in association with chlorine, the intake of sodium has been dependent upon the intake of sodium chloride. In the present investigation we have studied sodium deficiency as affecting the excretion of nitrogenous compounds, and as affecting the excretion of potassium, calcium, magnesium, phosphorus, sodium, and chloride, as respects both their absolute amounts and the pathways of their excretion.

### *Procedure*

Six young rats, litter mates, weighing 35 to 40 gm., were used. Three were placed on the experimental diet containing 0.002 per cent sodium, and the remaining three were fed the control diet, the sodium content of which was 0.66 per cent. The "paired" method of feeding was used. At the beginning of the experiment the rats were fed their respective diets for a period of 3 days in order to determine the food intake of the experimental animals, which served as the basis on which the initial food allowance of the control rats was determined, the daily intake of each control animal being restricted to that of the paired experimental rat. The caloric intake as well as all other dietary factors was thus equalized. The composition and preparation of the diets have

\* This work was aided by a grant from the Rockefeller Foundation.

been described previously (14). Distilled water was allowed *ad libitum*, and the water intake of each individual animal was recorded. The rats were weighed at the end of each metabolic period, which in this investigation was of 7 days duration. The balance studies were continued without interruption until the death of the last of the experimental rats, which occurred in the nineteenth period.

The procedure employed for the collection of the urine and feces and the methods used in the analysis of these excreta were essentially those described by Day and McCollum (2).

#### RESULTS AND DISCUSSION

The results reported here confirm the observations described in a previous publication (14) that the effects of sodium deficiency in the rat appear to be general in character rather than specific.

As reported previously, the control animals grew better than did their pair mates deprived of sodium. During the experimental period the average total body weight gain per rat of the animals on the sodium-deficient diet was 57.7 gm. and that of the controls was 97.5 gm. The average amount of water consumed was 1074 ml. and 1720 ml. respectively. The average amount of food eaten was 435 gm. and 452 gm. respectively. The amount of food consumed per gm. of body weight was 7.5 gm. and 4.6 gm. respectively.

The nitrogen balance figures (Table I) indicate that the rats deficient in sodium did not have the ability to utilize protein as efficiently as the controls. The sodium-deficient rats retained only 760 mg. per rat during the entire 19 weeks, whereas the average total retention of the controls was 2095 mg. The retention of the deficient animals was fair during the first 6 weeks. At about this time, when the first eye symptoms began to appear (14), the retention progressively decreased till the 14th week, when the eyes of the sodium-depleted rats were markedly affected and the animals showed a rapid loss of weight (14). At this time a negative nitrogen balance ensued and continued until death. This is in agreement with the findings of Schoorl (17), who fed a sodium-low diet to pigs and rats, and Sjollem (18), who kept chicks on a sodium-low diet and observed a low nitrogen balance.

Since the water consumption of the control animals in this study, as indicated, was markedly greater and the control animals had

good deposits of fat, whereas the experimental animals were markedly lean, as was described in an earlier publication (14), the difference in weight between the sodium-depleted and the control animals is, probably, due also to the difference in water retention and fat synthesis, as well as in the nitrogen balance, which is in accord with the observations reported by Kahlenberg, Black, and Forbes (10).

It is not entirely unexpected that the calcium, phosphorus, and chloride excretion in the two groups of rats does not show any considerable difference. Of particular interest is the metabolism of potassium in the sodium-deficient rats. In these animals the retention of potassium is considerably increased, being 310 mg., as contrasted with the control animals, whose retention was 78 mg. (Table I). The magnesium values indicate a somewhat greater retention of this mineral by the sodium-deficient rats, being 85 mg., whereas that of the control animals was 65 mg. These results suggest that under the experimental conditions described here the sodium-depleted animal apparently redistributes its basic elements in order to maintain acid-base equilibrium and water balance.

In the control animals the sodium values representing weekly balances over a 19 week experimental period show that when the animal organism is supplied with adequate amounts of sodium, the retention fluctuates considerably during the various metabolic intervals. In sharp contrast are the results of the depleted rats in which, although the sodium retention is negative, the sodium values are practically constant throughout the experimental period. It is evident that sodium is the only element studied here which undergoes marked change; its balance is negative from the very beginning of the deficiency (Table I), and, although the sodium-low diet was acid-forming, the alkaline reserve of these experimental animals was apparently not endangered, since there were no signs of an acidic condition. This confirms the observations that the rat is very resistant to any disturbance of the neutrality-regulating mechanism of the body (11, 13).

It is well established that to maintain the electrolytic concentration of the body fluids, both extracellular and intracellular, the sum of the basic elements must balance the sum of the acidic elements, for a reduction or increase in the total electrolytes, which comprise chiefly the basic radicals Na (extracellular) and

TABLE I

*Mineral Metabolism of Rats Fed Diet Extremely Deficient in Sodium Compared with Rats on Same Diet Supplemented with Sodium*

Min- eral	Metabolic balance	Period No.*																		Total		
		1		2-3†		4-5		6-7		8-9		10-11		12-13		14-15		16-17			18-19	
		Diet‡		Diet		Diet		Diet		Diet		Diet		Diet		Diet		Diet			Diet	
		54	55	54	55	54	55	54	55	54	55	54	55	54	55	54	55	54	55		54	55
		mg.		mg.		mg.		mg.		mg.		mg.		mg.		mg.		mg.		mg.		
Na	Intake	1	222	1	160	1	161	1	160	1	170	1	194	1	227	1	125	1	146	1	200	
	Output, urine	4	105	2	100	2	108	5	100	6	115	6	119	3	138	4	90	8	116	8	135	
	" feces	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
K	Retention	-4	116	-2	59	-2	52	-4	60	-5	55	-5	75	-2	89	-3	35	-7	30	-7	65	
	Intake	370	390	280	297	283	313	282	338	300	323	342	384	399	222	220	233	257	235	352	2995 3105	
	Output, urine	225	336	285	307	243	275	179	203	290	290	272	334	351	388	285	227	272	303	222	322	
Ca	" feces	1	1	2	1	2	2	6	5	15	10	7	8	17	9	9	4	1	1	1	1	
	Retention	144	53	-7-28	52	6	128	74	33	0	44	0	16	2	-72	-11	-40	-47	12	29	310 78	
	Intake	370	389	280	280	297	282	313	282	338	247	323	343	384	399	222	220	233	256	235	352	
P	Output, urine	6	12	15	30	17	21	14	18	41	28	15	30	17	22	16	17	12	25	41	42	
	" feces	47	70	73	64	125	99	165	137	166	120	174	178	199	206	177	127	131	197	174	267	
	Retention	317	307	192	186	155	162	134	127	131	99	134	135	168	171	29	76	90	34	20	43	
Mg	Intake	191	201	144	144	155	145	161	145	174	153	166	176	198	205	114	113	120	132	121	181	
	Output, urine	17	25	17	23	22	25	26	30	32	30	40	39	32	40	33	37	38	40	40	34	
	" feces	12	20	34	28	58	44	75	63	79	71	69	88	96	96	81	73	63	89	66	106	
	Retention	162	156	93	93	75	76	60	52	63	52	57	49	70	69	0	0	19	3	15	41	
	Intake	47	49	35	35	38	36	40	36	43	38	40	43	49	50	28	28	30	32	30	45	
	Output, urine	12	17	19	21	19	20	19	22	27	29	21	27	20	26	18	20	17	21	22	26	
	" feces	5	6	14	11	9	6	12	10	10	7	9	6	14	19	10	6	10	11	8	16	
	Retention	30	26	2	3	10	10	9	4	6	2	10	10	15	5	0	2	3	0	0	3	

Cl	Intake	187	197	142	142	150	143	158	143	171	152	163	173	194	202	112	111	118	130	119	173	1514	1566
	Output, urine	119	142	135	142	142	127	141	120	153	139	137	117	151	183	96	107	110	93	113	125	1297	1295
	feces	2	4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	4	6
N	Retention	66	51	7	0	8	16	17	23	18	13	26	56	43	18	15	3	8	37	3	48	211	265
	Intake	955	1005	724	724	766	729	807	728	872	851	833	882	934	991	573	577	602	713	404	908	7470	8108
	Output, urine	522	418	477	402	653	557	738	623	651	580	736	616	794	609	551	553	717	662	563	620	6402	5640
	feces	34	32	34	32	42	35	39	47	36	53	33	75	34	26	29	23	29	39	32	43	308	373
	Retention	433	587	213	290	71	137	30	58	185	218	64	191	106	356	-7	1	-144	12	-191	245	760	2095

\* Each period is 7 days.

† Values for each diet group are averages from three rats and two periods.

‡ Diet 54, diet deficient in sodium; Diet 55, deficient diet supplemented with sodium.



K (intracellular) and the acid radicals  $\text{HCO}_3$  and Cl, is accompanied by a corresponding and respective increase or decrease in body water. The reason for the difference in the water consumption of the sodium-deficient animals and those receiving sodium is easily explained, since it is well known that changes in the base and water content of the organism parallel one another. Gamble and his associates (3, 5, 7) have demonstrated that conditions under which the water content of the body is reduced lead to the excretion of an equivalent amount of base and, conversely, conditions which deplete base are attended by equivalent losses of water (4, 6, 8). Thus retention of base entails retention of water, and accumulation of water is associated with storage of base. When the water changes affect chiefly the extracellular fluids, the base simultaneously retained or lost is chiefly sodium. Sodium appears to be directly related to the water content of tissues, for water is not retained by the tissues unless they are supplied with sodium in spite of the fact that there is an abundance of chloride. Here again is pointed out the fact that the relation of sodium to water metabolism is close. The chloride metabolism of the sodium-low diets was apparently not disturbed in spite of the close physiological relationship between sodium and chloride (15, 16).

A physiological relation between sodium and potassium has been postulated by Bunge (1) and others (9, 12, 19) that in normal animals large intake of sodium causes excretion of potassium; the results in this study suggest that in case of small intake of sodium there is retention of potassium.

It appears, then, that, despite a limitation of sodium in the diet, severe enough to retard growth and impair the function of the animal organism, as described earlier (14), and despite an unbalance in the diet between base and acid, the sodium-depleted animal is sufficiently compensated by the increased retention in potassium and magnesium for neutralization of the metabolic acids excreted in the urine, and hence the delicate mechanism in the organism regulating the osmotic pressure and acid-base balance is not disturbed appreciably.

#### SUMMARY

The effects of a diet extremely deficient in sodium on the metabolism of the rat were studied over a period of 19 weeks. Bal-

ance determinations of sodium, potassium, calcium, phosphorus, magnesium, chlorine, and nitrogen in both urine and feces were made at 7 day intervals throughout the experimental period. There was a continuous negative sodium balance. The calcium, phosphorus, and chlorine metabolism did not seem to be affected. There was some indication of an increased retention of magnesium. The most striking effect of the deficiency was on the potassium, which showed a marked retention. The nitrogen retention, although progressively decreasing till the 14th week of the experiment, was positive. From that time on, *i.e.* the 14th week, it became negative simultaneously with the extreme pathological changes of the eyes and the onset of rapid loss of weight.

Rebecca Snyder, Helen Diering, and Betty Eckenrode made the analytical determinations.

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# HYALURONIC ACID IN THE PLEURAL FLUID ASSOCIATED WITH A MALIGNANT TUMOR INVOLVING THE PLEURA AND PERITONEUM\*

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Mucilaginous tumors and tumor fluids occur quite frequently. The nature of the mucilaginous constituent, however, has been determined in only one instance: Kabat (2) isolated a viscous polysaccharide acid from the tissues and a cyst of a tumor of a filtrable fowl sarcoma. This polysaccharide was shown by its chemical properties and by enzymatic hydrolysis to be very similar to, if not identical with, hyaluronic acid which has been found in cattle and pig vitreous humor (3, 4), human umbilical cord (3), cattle and human synovial fluid (5), and Group A hemolytic streptococci in the mucoid phase (6). Probably the mucoid capsule of Group C hemolytic streptococci also contains hyaluronic acid (7).

In this paper the isolation of hyaluronic acid from the viscous pleural fluid of a patient with a malignant tumor of the pleura and peritoneum is reported. The fluid was obtained through the courtesy of Dr. R. Loeb of the Department of Medicine. Clinically and pathologically the tumor was diagnosed as a mesothelioma or endothelioma. The peritoneal fluid had the same appearance as the chest fluid. It is quite obvious that the tumor cells themselves are the source of the hyaluronic acid, for this type of tumor frequently produces viscous fluids in the serous cavities (8). Furthermore, the usual transudates and exudates of the pleura and peritoneum are not viscous, even when a malignant tumor exists in these regions. Another indication is the almost constant

\* A preliminary report of this work has been published (1).

yield of hyaluronic acid in the fluids taken at different time intervals.

# EXPERIMENTAL

Three different samples of pleural fluid were obtained. The procedure used for the isolation of the polysaccharide acid was similar to that used for the isolation of hyaluronic acid from synovial fluid (5), except that a final precipitation of the material in 10 per cent acetic acid by 2 volumes of alcohol was substituted for the glacial acetic acid precipitation previously used. There-

TABLE I

*Analysis of Polysaccharide Acids Isolated from Pleural Fluid (Samples 1 to 3) and Cattle Synovial Fluid Corrected for Ash and Moisture*

Sample No.	Nitrogen	Hexosamine	Uronic acid	Acetyl	Acid equivalent weight	$[\alpha]_D$	Moisture	Ash	Equivalents per equivalent weight			
									Nitrogen	Hexosamine	Uronic acid	Acetyl
	per cent	per cent	per cent	per cent		degrees	per cent	per cent				
1	3.21	39.6	44.9	9.85	622	-76.8	1.27	4.04	1.43	1.38	1.44	1.43
2	3.00	40.8	46.6	10.5	576	-70.3	6.67	2.16	1.23	1.31	1.38	1.40
3	2.95	40.0	46.7	10.3	641	-67.7	3.15	1.44	1.35	1.43	1.54	1.53
Synovial*	3.42	39.1	42.5	10.2	477	-73.3			1.17	1.04	1.04	1.13

\* Average of six samples.

fore a mixture of the free acid and its calcium salt was obtained, as the analysis of the material and its ash showed. Table I gives the analyses of the three samples isolated compared with the average data of six samples of hyaluronic acid prepared from cattle synovial fluid.

The acid equivalent weights are too high and therefore the equivalents per equivalent weight are too high. But Table I shows that, in the limits of the error of the methods, the acid is composed of equimolar parts of hexosamine, hexuronic acid, and acetyl, and the rotation is very similar to that of the synovial fluid polysaccharide.

The hexosamine was isolated as the hydrochloride, as described earlier (3). 110 mg. were obtained from 300 mg. of polysaccharide or 82 per cent of the amount determined analytically. The analysis was as follows: N (Dumas) 6.43 per cent, Cl (by titration) 16.1 per cent (theoretical N 6.49 per cent, Cl 16.4 per cent), initial  $[\alpha]_D +103^\circ$ , at equilibrium  $+71.4^\circ$ . The compound was therefore glucosamine hydrochloride.

The identity of the pleural fluid polysaccharide with hyaluronic acid was finally established by enzymatic analysis. The enzyme was prepared from a rough pneumococcus designated as Strain D-39-R. Its preparation and properties are described elsewhere (9). The enzyme is highly specific for hyaluronic acid. The kinetics of hydrolysis of a sulfonated derivative of hyaluronic acid are, for example, quite different from that of the original sample.<sup>1</sup>

For comparison five different enzyme concentrations were incubated at  $37^\circ$  at pH 5.9, in the presence of toluene, with 5 mg. of the polysaccharides from the tumor fluid and from umbilical cord. Reducing values determined at 2 and 20 hours are plotted in Fig. 1. It is evident that the curves agree closely and from this agreement it is concluded that the polysaccharides are identical.

The yield of polysaccharide per 100 cc. of pleural fluid corresponded to 0.174, 0.187, and 0.142 per cent as compared to 0.02 to 0.025 per cent from bovine synovial fluid, about 0.04 per cent from bovine vitreous humor, and 0.130 per cent from the cyst fluid investigated by Kabat.

In previous papers from this laboratory it was pointed out that hyaluronic acid occurred free or in salt linkage only in vitreous humor and synovial fluid and was not chemically bound to protein (3, 10, 11). On the other hand, protein complexes are formed by the polysaccharide acids on acidification in the presence of protein. Such protein complexes were shown to be salts formed by the combination between the acid group of the polysaccharide and the basic groups of the proteins in stoichiometric proportions. The so called "mucins" prepared by acidification of the native fluid must therefore be considered artifacts.

<sup>1</sup> Only one polysaccharide acid not containing glucuronic acid has been found which is also split by the enzyme though at a markedly different rate. The latter was isolated from submaxillary gland (unpublished).

To test for the presence of protein compounds, samples of (a) the pleural fluid (Sample 1), (b) the polysaccharide isolated from it, (c) the polysaccharide from umbilical cord, and (d) an acid polysaccharide in a firm chemical linkage with a polypeptide<sup>1</sup> were submitted to Dr. Longworth of the Rockefeller Institute for electrophoretic analysis.<sup>2</sup>

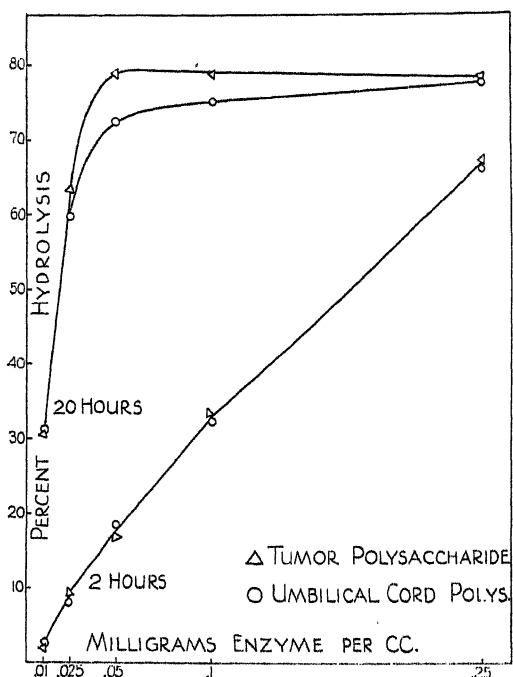


FIG. 1. Hydrolysis of polysaccharide acids by pneumococcus enzyme

The pleural fluid was centrifuged to clear it of cells, diluted with an equal volume of veronal buffer of pH 7.8, dialyzed against the buffer, and analyzed as described by Longworth and co-workers (12). Four components could be distinguished. The fastest component had a mobility of  $-10.5 \times 10^{-5}$ , and the second and main component a mobility of  $-5.6 \times 10^{-5}$ , which is similar to that of albumin in serum. Moreover, the yellow pigment of

<sup>2</sup> We are greatly indebted to Dr. Longworth for carrying out these experiments.

the exudate migrated with this second component. This is similar to the migration of bilirubin with serum albumin and suggests that the second component of the chest fluid is serum albumin. The third and fourth components have mobilities of  $-2.9$  and  $-0.5$  respectively, similar to the  $\beta$ - and  $\gamma$ -globulins of serum. No peak which might correspond to  $\alpha$ -globulin was evident in the pattern. This is probably due to the fact that, owing to the high mobility of the first component, the electrolysis was interrupted too early for this component to separate from the albumin. The total area of the pattern corresponded to the following values, first component 5 per cent, second component 65 per cent, third component 13 per cent, fourth component 17 per cent.

From the nitrogen value of the fluid, minus non-protein nitrogen, minus glucosamine nitrogen of the isolated polysaccharide, the total protein in the fluid was calculated as 3.55 per cent. From this figure and from the relative concentration of the first component, a concentration in the original fluid of this component was calculated as 0.17 per cent, while the amount isolated by us corresponded to 0.174 per cent. The close agreement between these figures indicates that there was migration of the free polysaccharide rather than of a protein complex. This is borne out by the mobility of the isolated polysaccharide acids at the same pH. The polysaccharides of the tumor and the umbilical cord both had a mobility of  $-10.7 \times 10^{-5}$ , while the peptide complex of an acid polysaccharide (which, however, contains a hexonic acid instead of glucuronic acid) had a mobility of  $-6.95 \times 10^{-5}$ . The close agreement between the mobility of the fast component in the tumor fluid and of the isolated polysaccharides seems to prove that the fast component in the fluid is the free polysaccharide acid.

Hyaluronic acid or its salts in solution always show a marked viscosity. However, the data on solutions from different sources and by different investigators have varied considerably. As a rule the viscosity varied inversely with the purity of the preparations. In the case of synovial fluid, though no component of marked viscosity could be found other than hyaluronic acid, nevertheless the isolated polysaccharide accounted for only a fraction of the viscosity of the fluid from which it was prepared.

A very great discrepancy was found in the present case. The relative viscosity of the native fluid centrifuged at 4000 R.P.M.



was 147.6 at 20° (relative to 0.9 per cent sodium chloride), while the neutralized polysaccharide at a corresponding concentration of 0.177 per cent in 0.9 per cent sodium chloride had a viscosity of only 1.54. (The viscosity of a 0.25 per cent solution, the concentration used in work previously published, was 1.79 (3, 5).) In Fig. 2 the relative viscosities of the pleural fluid have been plotted against the dilutions of the fluid with 0.9 per cent sodium chloride.

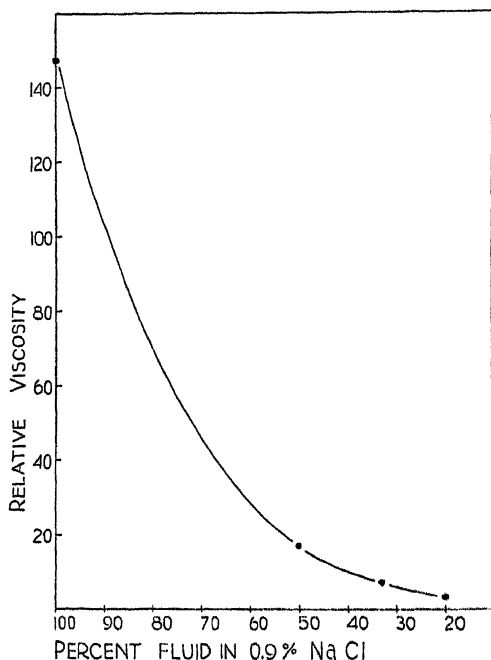


FIG. 2. Relative viscosity of pleural fluid at different dilutions

The great drop in viscosity obviously cannot be explained by the assumption of a true solution of fibrous macromolecules.

#### DISCUSSION

From the drop of the viscosity on dilution and the normal conductance of the dialyzed and diluted fluid (reported by Dr. Longworth), it must be concluded that the original fluid is a gel formed by the polysaccharide acid and the copresent protein. This con-

clusion is further borne out by the fact that the fluid shows thixotropy and that air bubbles in the fluid assume an ellipsoidal shape, both factors typical for a gel (13). The great variations in viscosity of the isolated polysaccharides may also best be explained by gel formation. The gel formation of the polysaccharide acid in the capsule of the hemolytic streptococci is obviously of great biological importance, as is in all likelihood the status of the polysaccharide acid in the vitreous humor.

A mobility similar to the one in the present case has been observed by Hesselvik in filtered vitreous humor (14). The fastest component had a mobility of  $-11.4 \times 10^{-5}$ , which the author ascribed to "hyalomucoid." The difference between this and the mobility of  $-10.5 \times 10^{-5}$  measured by Longworth is probably due to difference in buffers and in ionic strength. Obviously the Upsala investigator was likewise measuring the mobility of hyaluronic acid.

Hyaluronic acid in the capsule of hemolytic streptococci is connected with the virulence of the organisms. The virulent forms have been shown to possess mucoid capsules (15). Moreover the appearance in the organisms of an enzyme hydrolyzing hyaluronic acid is apparently somehow connected with the loss of virulence (9).<sup>3</sup> In Group C hemolytic streptococci good experimental evidence has been brought forward to link the amount of an immunologically inactive acid polysaccharide, which is apparently hyaluronic acid, with the degree of encapsulation, virulence, and invasiveness (7). Another example of the connection between bacterial virulence and a mucilaginous constituent is that of gastric "mucin" which renders virulent for mice (or increases the virulence of) a number of microorganisms avirulent (or of low virulence) for this species (16, 17). This effect of a mucoid capsule is obviously not due to any change inherent in the microorganism, but is probably due to a failure of the natural defense mechanism of the host to cope with the invading organism so that the latter can grow unchecked. It is tempting to speculate in the case of the malignant tumors as to the operation of a similar mechanism in which hyaluronic acid or similar constituents may prevent the action of the normal restraining influences of the host tissues, so

<sup>3</sup> Unpublished data.

that the inherent growth tendencies of the tumor tissues become dominant.

#### SUMMARY

The viscous component of a pleural fluid associated with a malignant tumor of the pleura and peritoneum has been isolated. The substance has been shown to be hyaluronic acid and to be similar in composition and rotation to this polysaccharide obtained from other sources. This was further substantiated by the results of the enzymatic hydrolysis of a pneumococcus enzyme.

In the electrophoresis apparatus a very fast component with a mobility of  $-10.5 \times 10^{-5}$  was observed and shown to be free hyaluronic acid. The concentration of this fraction as determined by the electrophoresis experiment was very similar to the concentration of the acid obtained by isolation. Furthermore, the mobility of hyaluronic acid isolated from the tumor fluid and from umbilical cord was  $-10.7 \times 10^{-5}$ , very similar to that of the fast fraction in the pleural fluid.

The viscosity of the tumor fluid, as measured in an Ostwald viscosimeter, was about 95 times greater than the viscosity of the isolated polysaccharide acid in an equivalent concentration, dropping sharply on dilution. By the abnormal viscosity the fluid is characterized as a gel. Additional evidence for this hypothesis is given.

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## ANAPHYLACTIC SHOCK AND NITROGEN METABOLISM IN THE DOG\*

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It has been found that the severity of the clinical response to an intravenous shock dose of horse serum in a previously sensitized normal dog is definitely paralleled by the increase in urinary nitrogen. When the clinical response is mild, there is little increase in the total urinary nitrogen and this increase is manifest mainly in the urea-ammonia fraction. When the clinical response is severe as indicated by a profound fall in blood pressure, prolonged bloody diarrhea, vomiting, and collapse, there is a very pronounced increase in total urinary nitrogen. This increase over a previously established base-line is most marked in the urea-ammonia fraction, but there are also significant increases in the creatine and uric acid fractions, while the creatinine shows little change.

Previous reports from this laboratory show that *tissue injury* in the dog produced by a sterile turpentine abscess or by prolonged chloroform anesthesia elicits certain typical changes in the excretion of urinary nitrogen. The normal dog in which a sterile abscess has been produced will show a great increase in urinary nitrogen above the level of the simple fasting dog (1), and there is likewise a great increase in urinary nitrogen in the 4 days following chloroform anesthesia (2). In both types of injury, creatinine excretion remained fairly constant, while the urinary creatine and uric acid excretion increased considerably during the acute stage of the injury.

In the course of experiments designed to study the effect of body protein stores on susceptibility to horse serum anaphylaxis, and the effect of the latter on plasma protein formation under

\* Aided by a research fund given by Eli Lilly and Company.

conditions of plasmapheresis, it was found of interest to examine the urinary nitrogen excretion of normal dogs in anaphylactic shock.

The *total* urinary nitrogen excretion during anaphylactic shock has been studied by a number of investigators (3-5) in the guinea pig, the rabbit, and the dog. In general they have concluded that there is an increased excretion of total urinary nitrogen. Major (6) has also stated that in the rabbit there is always an increase in urinary creatinine and that he was unable to find an anatomic foundation for these noteworthy changes in nitrogen metabolism.

### *Methods*

All dogs used in these experiments were active, healthy, adult females. Before the metabolism studies were started, all dogs were sensitized to horse serum by giving 5 cc. of horse serum subcutaneously followed within the next 2 days by 5 cc. of horse serum given intravenously. The experiments were then planned so that at least 21 days elapsed before the shock dose of 20 cc. of horse serum was given intravenously.

As indicated in the individual protocols, all dogs were fasted 2 to 4 days at the beginning of the metabolism study. They were then placed on a diet which was protein-free except for 2 to 3 gm. of a solid liver extract.<sup>1</sup> Under this régime, the urinary nitrogen excretion rapidly approached a uniform base-line level. During the metabolism experiments, the dogs were kept in galvanized iron metabolism cages where they were under constant supervision. The urine was collected frequently as passed and was preserved by toluene and refrigeration. Each 24 or 48 hour period was terminated by catheterization of the animal and rinsing of the bladder with water. The urine of each period was analyzed for the following constituents: total nitrogen by macro-Kjeldahl, ammonia and urea by aeration with the urease method, creatinine and creatine by the methods of Folin, and uric acid by the method of Morris and Macleod.

### EXPERIMENTAL

From the clinical history of Dog 37-230 (Experiment 1), it will be noted that only a *very mild* clinical reaction of anaphylactic

<sup>1</sup> We are indebted for this extract to Eli Lilly and Company.

shock was observed. Urinary nitrogen partition revealed no significant changes in any of the fractions.

*Experiment 1. Clinical Experimental History during Metabolism Study*

Dog 37-230. Adult female hound. This dog was given 5 cc. of horse serum subcutaneously, followed within 3 days by 5 cc. of horse serum intravenously about 4 weeks before the metabolism study began. In this 4 week interval, the dog was bled occasionally and maintained on the low protein diet (sucrose 118 gm., lard 27 gm., protein-free butter 12 gm., bone ash 3.3 gm., salt mixture 2.3 gm. (7), liver extract 2.4 gm., cod liver oil 15 gm.). At the start of the metabolism period, this dog was hypoproteinemic (plasma protein level 4.4 gm. per cent) and somewhat anemic (red cell hematocrit 30 per cent).

Aug. 31. Urine collection started. Weight 11.4 kilos.

Sept. 1-5. Ate an average of 60 to 70 per cent of the diet.

Sept. 6. Given 20 cc. of horse serum intravenously. Within 1 minute the pulse became very perceptibly weaker. The dog staggered about for 2 or 3 minutes, and then stood with legs bowed for about 15 minutes; occasional movements were weak and incoordinated. 20 minutes after serum, the dog moved on call but with some stiffness of the hind legs. After 45 minutes the pulse was firm, regular, and easily palpated and the dog was apparently completely recovered.

Sept. 6-11. Ate an average of 50 to 60 per cent of the diet. Returned to kennel in good condition. Weight 10.3 kilos.

*Nitrogen Metabolism*—With base-line excretion of 1.80 gm. of total urinary nitrogen, there were 1.57, 1.42, 1.64 gm. of total urinary nitrogen in the three 48 hour periods following the shock dose of horse serum. There were no significant changes in the creatinine, creatine, or uric acid excretion.

Table I presents data obtained from Dog 37-233 (Experiment 2). The clinical history reveals that the anaphylactic shock was relatively mild and that recovery was rapid. There is only a slight increase in the total nitrogen excretion occurring in Periods 4 and 5. The changes in creatinine and creatine are insignificant and there is a definite increase in the uric acid excretion in Periods 4 and 5.

In Table II are summarized data from Experiments 3 and 4, both carried out on the same dog (No. 37-178). In both experiments the clinical symptoms of anaphylactic shock were similar and of marked severity and duration. There are also parallel increases in urinary nitrogen excretion. In Periods 7 and 8 of both Experiments 3 and 4, there is a very large increase in total urinary nitrogen. This increase consists largely of urea and ammonia, the percentage of which rises accordingly. Although the cre-



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atinine excretion undergoes no striking change, there is a very remarkable increase in creatine nitrogen which amounts to 5 times the basal excretion. In Period 8 of Experiments 3 and 4, there is a

TABLE I

*Experiment 2. Fasting; Low Protein Diet; Mild Anaphylactic Shock*  
Dog 37-233.

Period No.	Period	Total N	Urea N + NH <sub>3</sub> -N		Creatinine N		Creatine N		Uric acid N
		mg.	mg.	per cent	mg.	per cent	mg.	per cent	
1	48	2130	1390	65.2	186	0.9	36	0.2	4
2	48	1940	1250	64.4	155	0.8	77	0.4	4
3	48	2390	1600	66.9	195	0.8	69	0.3	5
20 cc. horse serum intravenously									
4	24	1590	1040	65.3	122	0.8	33	0.2	7
5	24	1410	900	63.8	105	0.8	12	0.1	7
6	48	2190	1440	66.8	178	0.8	6	0.05	4
7	48	2270	1480	65.2	194	0.9	53	0.2	5
8	48	2300	1450	63.0	177	0.8	47	0.2	4

*Experiment 2. Clinical Experimental History during Metabolism Study Period (Table I)*

Dog 27-233. Adult female bull dog.

May 28. 5 cc. of horse serum subcutaneously.

May 30. 5 cc. of horse serum intravenously.

June 21. Fasting started which was continued through June 24.

June 25. Low protein diet started, which was eaten regularly and completely throughout the experiment. The diet was the same as that of Dog 37-230, Experiment 1.

July 6. Urine collections started. Weight 10.7 kilos.

July 12. Horse serum (20 cc.) intravenously. Symptoms of distress within 3 minutes with pronounced weakening of the pulse, followed by violent vomiting, defecation, irritation about the muzzle, and congested conjunctivae. 1 hour after horse serum, the condition was very much improved and the dog ate the regular diet completely within the next 24 hours.

July 20. Dog in excellent condition. Weight 10.9 kilos. Returned to kennel.

significant increase in uric acid. Incidentally, both experiments demonstrate very nicely the protein-sparing action of a carbohydrate-rich diet after Periods 1 and 2 of fasting.

The results of Experiment 5, shown in Table III, are practically

identical with those of Experiments 3 and 4. Here again a severe clinical reaction is followed by correspondingly severe changes in urinary nitrogen excretion. The increased excretion of nitrogen starts sooner than in Experiments 3 and 4, although in all these

TABLE II

*Fasting; Low Protein Diet; Severe Anaphylactic Shock*

Dog 37-178.

Period No.	Period	Total N	Urea N + NH <sub>3</sub> -N	Creatinine N	Creatine N	Uric acid N
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Experiment 3									
	hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.
1	48	4500	3880	86.3	168	3.7	60	1.3	15
2	48	3570	2850	80.0	180	5.0	18	0.5	18
3	48	2020	1340	66.4	169	8.3	9	0.4	15
4	48	2960	2180	73.5	157	5.3	31	1.0	13
5	48	2420	1720	71.0	156	6.5	27	1.1	13

20 cc. horse serum intravenously									
6	24	1320	930	70.0	46	3.4	43	3.3	5
7	24	6000	5680	93.4	84	1.4	203	3.4	7
8	48	3540	2690	76.0	130	3.7	113	3.2	22
9	48	1870	1200	64.4	116	6.2	20	1.1	18
10	48	1480	820	55.5	116	7.8	26	1.8	18
11	48	1560	900	57.8	116	7.4	17	1.1	14
12	48	1530	860	56.4	110	7.2	12	0.8	14

Experiment 4									
1	48	4830	4120	85.3	138	2.9	12	0.3	12
2	48	3920	3220	82.2	71	1.8	12	0.3	11
3	48	1870	1260	67.5	133	7.1	30	1.6	9
4	48	1840	1130	61.4	125	6.8	21	1.1	13
5	48	2090	1360	64.8	130	6.2	36	1.7	19

20 cc. horse serum intravenously									
6	24	1050	620	59.0	47	4.5	26	2.5	5
7	24	4410	3730	84.7	73	1.7	173	3.9	14
8	48	4570	3650	79.9	110	2.4	168	3.7	22
9	48	1790	1180	65.9	102	5.7	25	1.4	13
10	48	1770	1170	65.9	105	5.9	5	0.3	13
11	48	1790	1180	65.9	103	5.8	10	0.6	12

TABLE 11—*Concluded**Experiment 3. Clinical Experimental History during Metabolism Study Period (Table II)*

Dog 37-178. Adult female hound mongrel.

Apr. 18. 5 cc. of horse serum subcutaneously.

Apr. 20. 5 cc. of horse serum intravenously.

Apr. 28-May 3. Fasted. Water in cage at all times. Urine collection started Apr. 30.

May 4. Daily low protein diet started (sucrose 96 gm., lard 19 gm., protein-free butter 9 gm., bone ash 3 gm., salt mixture 2 gm. (7), liver extract 2 gm., cod liver oil 15 gm.). Weight 8.5 kilos.

May 11. Given 20 cc. of horse serum intravenously. Followed almost immediately by marked weakening of the pulse, severe vomiting, and diarrhea. Dog unable to stand; had convulsive running movements. Collapsed completely within 30 minutes. Given 1 cc. of adrenalin in 1:10,000 intramuscularly. Vomiting continued at intervals and vomitus became bile-stained. Diarrhea bloody. The dog was somewhat brighter later in the day and was able to stand weakly. Refused food.

May 12. Moderate bloody diarrhea continued and contaminated urine. Otherwise much improved. The dog ate 50 per cent of the diet.

May 13. Condition better. The dog ate 50 per cent of the diet.

May 14-22. Dog in good condition. Ate 100 per cent of the diet. Weight 7.8 kilos.

*Experiment 4*

Dog 37-178.

July 10-13. Fasted. Urine collection started July 11.

July 14. Low protein diet started (same as Experiment 3). The dog ate the diet regularly and completely until July 21. Weight 8.1 kilos.

July 21. Given 20 cc. of horse serum intravenously. Response very similar to that seen in Experiment 3. The dog ate no food.

July 22. The condition was much improved, but the dog ate no food (very hot day).

July 23. The condition was very good, but the dog ate no food.

July 24. The condition was very good. The dog ate 75 per cent of the diet.

July 25-30. Ate 100 per cent of the diet. Returned to kennels in good condition. Weight 7.5 kilos.

experiments the maximum effect is seen in the second 24 hour period following the shock dose of horse serum. The increased creatine excretion is just as striking as that seen in Experiments 3 and 4, and the increased uric acid excretion is even more pronounced.

TABLE III

*Experiment 5. Fasting; Low Protein Diet; Severe Anaphylactic Shock*  
Dog 38-18.

Period No.	Period	Total N	Urea N + NH <sub>2</sub> -N		Creatinine N		Creatine N		Uric acid N
	hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.
1	48	4530	3520	77.7	77	1.7	19	0.4	10
2	48	4240	3200	75.4	103	2.4	72	1.7	8
3	48	3410	2480	72.9	111	3.3	54	1.6	8
4	48	3570	2470	69.2	184	5.2	92	2.6	7
5	48	3400	2880	69.9	212	6.2	69	2.0	10

20 cc. horse serum intravenously

6	24	2900	2090	72.1	100	3.5	91	3.1	5
7	24	5470	4220	77.2	139	2.5	223	4.1	26
8	48	3840	2650	69.0	202	5.3	81	2.1	41
9	48	3640	2480	68.3	196	5.4	28	0.8	20
10	48	3650	2480	68.0	192	5.3	19	0.5	17
11	48	2640	1720	65.0	162	6.1	24	0.9	12
12	48	2600	1640	63.9	171	6.6	34	1.3	10

*Experiment 5. Clinical Experimental History during Metabolism Study*  
(Table III)

Dog 38-18. Adult female hound mongrel.

Sept. 19. 5 cc. of horse serum subcutaneously.

Sept. 21. 5 cc. of horse serum intravenously.

Sept. 30-Oct. 2. Fasted. Urine collection started on Oct. 1.

Oct. 3. Low protein diet started (same as diet of Dog 37-230, Experiment 1). Weight 12.5 kilos.

Oct. 3-12. The dog ate 100 per cent of the diet regularly.

Oct. 13. Given 20 cc. of horse serum intravenously. Defecated almost immediately, and attempted to defecate again after vomiting and retching for 4 minutes. Pulse very weak, barely palpable. Dog collapsed. Given 0.5 cc. of 1:10,000 adrenalin intramuscularly. Later some bloody diarrhea, and bile-stained vomitus. Since the dog appeared very weak, it was given several doses of 5 per cent glucose in saline (total 400 cc.). Ate none of the diet.

Oct. 14. Looked brighter, but still weak. Given 200 cc. of 5 per cent glucose by vein. Ate 60 per cent of the diet.

Oct. 15. Very much better.

Oct. 16-24. Dog in good condition. Returned to kennels. Weight 12.1 kilos.

## DISCUSSION

From the above experiments, we can conclude that the changes in urinary nitrogen excretion in anaphylaxis are very similar to those observed in dogs suffering from a discrete, readily demonstrable tissue injury. Exactly such changes have been described in dogs with sterile turpentine abscesses, and in dogs with liver injury from chloroform anesthesia. In the normal dog, an augmented urinary nitrogen excretion with increased creatine and uric acid excretion is very probably a general response to extensive tissue injury of any type, be it obviously demonstrable, or of a more subtle systemic sort as in anaphylaxis.

The above conclusion is in accord with the views expressed recently by Moon (8); namely, that the anaphylactic reaction is cellular rather than humoral, and that the meeting of antibody and antigen within the cells *irritates* or *injures* them. He adds that, if the *injury* is severe, it results in an inflammatory reaction not different from that which follows other injuries.

The large increase in urinary creatine suggests that there is extensive damage to muscle tissue. It is a well known fact that the response of smooth muscle tissue is involved in the early acute stage of anaphylactic shock. It is not unreasonable to suspect that this is at least partly responsible for the increased creatine excretion.

The fact that uric acid excretion increases following a moderately severe anaphylactic shock suggests that the liver function of converting uric acid to allantoin is somewhat impaired. This may be a result of the severe congestion and possible injury noted (8) in the liver after anaphylactic shock in the dog.

## SUMMARY

Anaphylactic shock in dogs results in increased urinary nitrogen excretion paralleling in severity the clinical symptoms of the anaphylactic shock. This response is strictly comparable with that resulting from very obvious tissue injury, and substantiates the view that anaphylactic shock may be marked by tissue injury of considerable severity.

The increased creatine and uric acid excretion following anaphylactic shock suggests and supports the contention that both muscle and liver tissue undergo injury in anaphylactic shock.

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## MICELLE FORMATION IN AQUEOUS SOLUTIONS OF BILE SALTS

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The solubility of fatty acids and cholesterol in bile does not appear to be due to the formation of soluble compounds of the choleic acid type. The bile is made up chiefly of the conjugated bile salts and such compounds with conjugated bile acids or salts have not been isolated (1). It is generally agreed that the solution of oils in aqueous solutions of soaps is associated with the micelle formation of the soap, the oil being dissolved, most likely, in the lipid center of the micelle (2-4). The bile salts are similar to the soaps in that they consist of a relatively large hydrophobic group and an ionic group. They might be expected also to form micelles and thus to be able to take up water-insoluble substances in a manner comparable to that of the soaps.

Bashour and Bauman (5) were unable to find a sharp change of slope in the equivalent conductivity-concentration curve of sodium desoxycholate, usually taken as an indication of micelle formation, although freezing point determinations on the more concentrated solutions indicated some degree of aggregation. It seemed likely that measurement of the osmotic coefficient as well as of the equivalent conductivity at various concentrations would offer some evidence as to the state of aggregation of bile salts.

### EXPERIMENTAL

Cholic acid was obtained from the Sandoz Chemical Works, Inc. Its moisture content was determined as 2.2 per cent. Glycocholic and glycodesoxycholic acids were prepared by the methods of Cortese and Bauman (6). Sodium oleate was obtained from

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Merck and Company, Inc., and used without further purification. The preparation gave a slightly cloudy solution at the higher concentrations (0.19 to 0.43 *m* per liter). The bile acids were dissolved in 0.5 *N* NaOH and adjusted to pH 7.8 to 8.0. The more dilute solutions were obtained by adding various amounts of water to the original solution. The solutions were made up by weight and, from density measurements, the concentration was expressed both on a weight and volume basis. In calculation of the osmotic coefficient, the concentration was expressed as moles of salt per kilo of water, whereas in the calculation of equivalent conductivity the concentration was expressed as moles of salt per liter of solution.

By means of the thermoelectric method as modified by Baldes (7) and by Baldes and Johnson (8), the vapor pressures of the bile salt solutions were compared with those of reference NaCl solutions. The osmotic coefficients of the bile salts were then calculated from the osmotic coefficients of NaCl which were derived from the freezing point data given in International Critical Tables (9). Calculations were made by means of the relation  $g_b = (m_{\text{NaCl}} \times g_{\text{NaCl}})/m_b$ , where  $g_b$  is the osmotic coefficient of the bile salt at the concentration  $m_b$ ,  $m_{\text{NaCl}}$  is the concentration of NaCl solution which has the same vapor pressure as the bile salt solution, and  $g_{\text{NaCl}}$  is the osmotic coefficient of sodium chloride at that concentration, the concentrations being expressed as moles per kilo of water. The osmotic coefficient of sodium chloride was calculated from the relation,  $g = \Delta t / (2 \times 1.858 \times m)$ , where  $\Delta t$  is the lowering of the freezing point and  $m$  is the molality. Several measurements with four to six thermocouples permit the determination of the vapor pressure of a solution with an accuracy equivalent to 0.0001 to 0.0002 mole of NaCl per kilo of water. Hence, the determination of the osmotic coefficient can be made with a fair degree of accuracy at a concentration as low as 0.005 *m*.

Conductivity measurements were made with a Wheatstone bridge obtained from Leeds and Northrup, a Kohlrausch slide wire and a microphone hummer being used. The conductivity cell was made of Jena glass and had a cell constant of 0.5674 cm.<sup>-1</sup>. Since the water used in making up the solution had a relatively high conductivity ( $2.5 \times 10^{-6}$ ), conductivity measurements were made only on dilutions down to 0.003 *m*. All measurements were made at a temperature of 25°.

*Results and Comment*

From the results of the measurement of osmotic activity and of equivalent conductivity it is evident that the bile salts which were studied undergo micelle formation in aqueous solutions. In the case of sodium glycocholate (Fig. 1) the osmotic coefficient is decreased by 50 per cent as the concentration is increased from 0.0047 M to 0.098 M, while the equivalent conductivity is decreased only 19 per cent over the same range of concentration, thus indi-

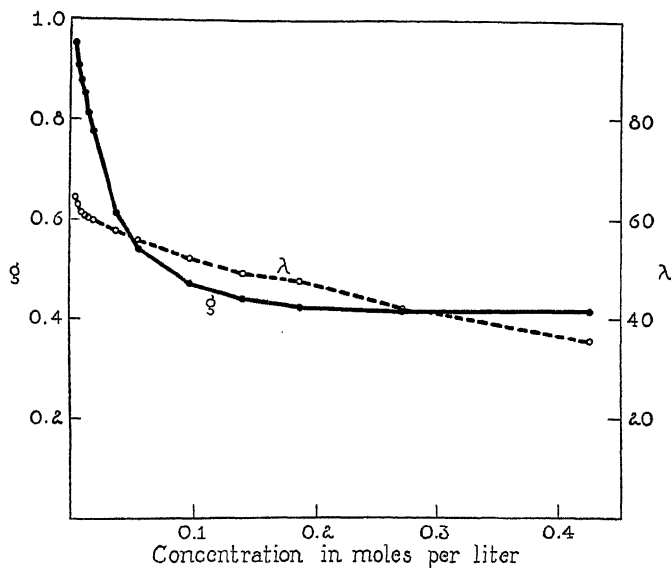


FIG. 1. Osmotic coefficient ( $g$ ) and equivalent conductivity ( $\lambda$ ) of sodium glycocholate at 25°.

cating an appreciable association of the bile acid ions. With sodium glycodesoxycholate (Fig. 2) the osmotic coefficient is decreased by 56 per cent and the equivalent conductivity by 19 per cent as the concentration is increased from 0.0047 to 0.093 M. Similar results were obtained with solutions of sodium cholate (Fig. 3).

The results obtained with the bile salts differ somewhat from those obtained with sodium oleate (Fig. 4). In the range of concentration in which micelle formation of sodium oleate occurs,

there is a sharp drop in the equivalent conductivity as well as in the osmotic coefficient. This marked decrease in equivalent conductivity with increase in concentration appears to be true generally for the paraffin chain salts and is the result of decreased ionization due to the local concentration of the ions making up the micelle (2). With sodium glycodesoxycholate (Fig. 2), however, the equivalent conductivity remains nearly constant over a short range of concentration in which the osmotic coefficient decreases markedly. This difference might be explained by

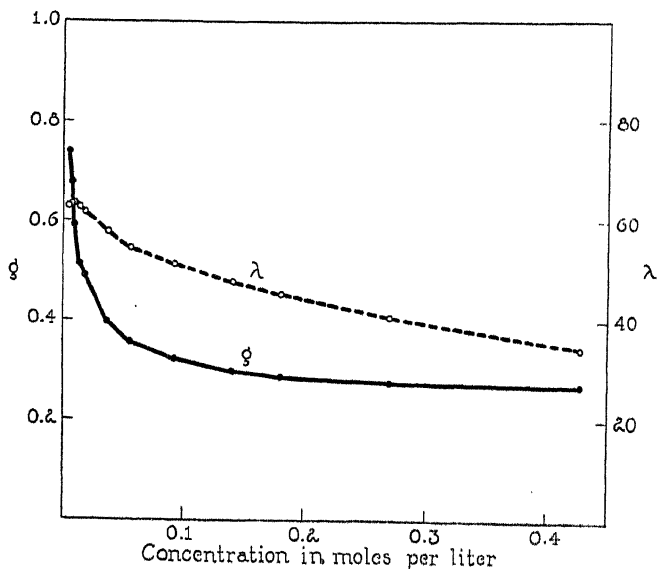


Fig. 2. Osmotic coefficient ( $g$ ) and equivalent conductivity ( $\lambda$ ) of sodium glycodesoxycholate at 25°.

postulating that, at least at the lower concentrations, the bile salt micelles consist, on an average, of a smaller number of anions than do the soap micelles. However, definite conclusions concerning this difference between the bile salts and soaps are not justified with the data at hand.

The slight initial rise in the osmotic coefficient curves for sodium cholate and sodium oleate (Figs. 3 and 4) is probably due to experimental error. Measurements on these solutions were made at concentrations down to 0.003 M at which concentrations the error

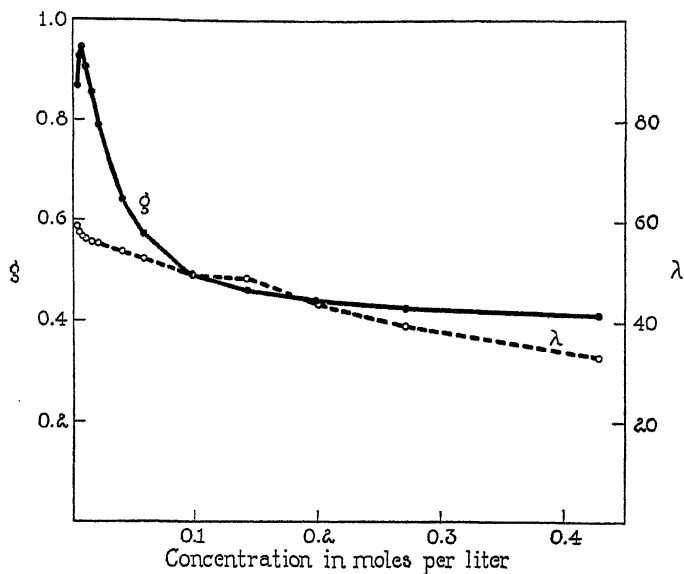


FIG. 3. Osmotic coefficient ( $g$ ) and equivalent conductivity ( $\lambda$ ) of sodium cholate at 25°.

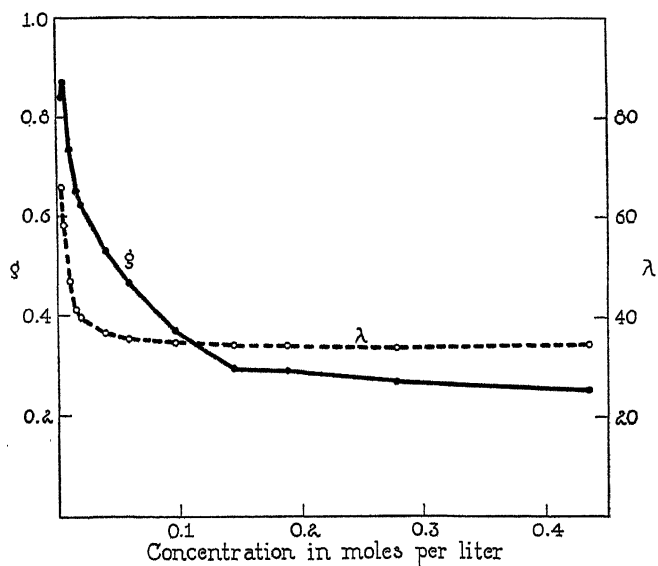


FIG. 4. Osmotic coefficient ( $g$ ) and equivalent conductivity ( $\lambda$ ) of sodium oleate at 25°.

in the determination of the osmotic concentration may be appreciable. With sodium glycocholate and sodium glycodesoxycholate, osmotic coefficient measurements were made at concentrations of not less than about 0.005 M.

While it cannot be definitely stated at this time that the solution of water-insoluble substances by the bile is associated with aggregation of the bile salt ions, such a conclusion appears reasonable. Bashour and Bauman (5), using pure solutions of the bile salts at 37.3°, found that the amount of cholesterol dissolved per gm. of bile salt increased with the concentration of the bile salt. In the case of sodium glycodesoxycholate, the weight of cholesterol dissolved per gm. of bile salt increased to a constant value of 40 mg. at a concentration of about 3 per cent (0.064 M) of bile salt. As seen from Fig. 2, this is in the range of concentration at which the slope of the osmotic coefficient curve of this salt is rapidly decreasing. It would be of interest to compare the osmotic coefficient-concentration curves of the various bile salts with their solvent properties at the same temperature and pH.

#### SUMMARY

Osmotic coefficient-concentration and equivalent conductivity-concentration curves were determined for sodium glycocholate, sodium cholate, sodium glycodesoxycholate, and sodium oleate at 25°. The results indicate that the bile salts form ionic micelles above a critical concentration. It is suggested that solution of water-insoluble substances by the bile may be associated with micelle formation of the bile salts in a manner similar to the solution of oils by soap solutions.

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**SYNTHESIS AND DETERMINATION OF THE LIPOTROPIC  
ACTIVITY OF THE BETAINES HYDROCHLORIDES OF  
*dl*-SERINE, *dl*-THREONINE, AND  
*dl*-ALLOTHREONINE\***

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(Received for publication, November 20, 1939)

This paper reports the synthesis of the betaine hydrochlorides of serine, threonine, and allothreonine and a study of the action of these substances on the lipid content of the livers of rats fed a high fat, low protein diet. This work was undertaken for two purposes. Best and Huntsman (1) found that choline prevented the development of fatty livers in rats fed a high fat diet and that betaine possessed a similar lipotropic effect. Since choline contains a hydroxyl group, it was of interest to determine the lipotropic activity of some hydroxybetaines which are closely related to choline in structure.

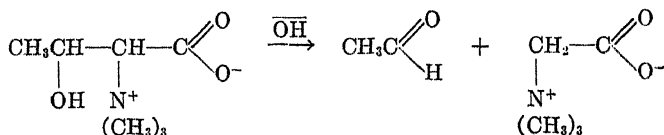
It also seemed possible that these studies might throw some light on the origin of choline in the animal body. The discovery of Channon and Wilkinson (2) and of Best and Huntsman (3) that casein has a lipotropic effect has been interpreted as indicating the possible synthesis of choline from one of the constituent amino acids of casein. Of the amino acids of casein, serine, containing the hydroxyethylamine grouping, is most closely related structurally to choline. Hence the physiological behavior of serine betaine is of particular interest, although it represents only one of

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† The experimental data in this paper are taken from a thesis submitted by Donald B. Melville in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

the possible intermediates by which serine might be converted into choline *in vivo*.

In the course of the synthetic work it was discovered that the betaines of threonine and allothreonine (but not of serine) are unstable in alkaline solutions, undergoing a retrograde aldol condensation as shown in the accompanying equation.



Preliminary experiments indicate that this reaction offers promise as a method for the quantitative determination of threonine. A study of other hydroxybetaines has revealed the fact that this reaction is a general one for  $\alpha$ -amino- $\beta$ -hydroxy acids and may be useful in differentiating these compounds from the isomeric  $\beta$ -amino- $\alpha$ -hydroxy acids.

#### EXPERIMENTAL

##### *Preparation of Betaine Hydrochlorides*

The preparation of the betaines was first attempted by the method of Novak (4). 11.9 gm. of *dl*-allothreonine, dissolved in 100 cc. of 1.0 N potassium hydroxide, were methylated with 56.7 gm. (0.45 mole) of dimethyl sulfate and 100 cc. of 4.5 N potassium hydroxide. The alkaline reaction mixture was then refluxed in order to destroy any unchanged dimethyl sulfate. The solution immediately became hazy and a dark brown precipitate gradually separated. At the end of 30 minutes the mixture was cooled and filtered. The solution had a strong odor of acetaldehyde. The filtrate was decolorized with norit and worked up in the usual manner. The crude product was recrystallized from alcohol, giving 5.0 gm. of a crystalline compound melting at 236–238°. The analytical data indicated that this substance was betaine hydrochloride instead of the expected allothreonine betaine hydrochloride.

$\text{C}_7\text{H}_{16}\text{O}_3\text{NCl}$ .	Calculated.	N 7.09, Cl 17.97
$\text{C}_6\text{H}_{12}\text{O}_2\text{NCl}$ .	"	" 9.11, " 23.13
	Found.	" 9.07, " 23.47

The identity of the substance was confirmed by the preparation of the picrate which melted at 180–182° and gave no depression of the melting point when mixed with an authentic sample of betaine picrate. Evidently allothreonine betaine is unstable in an alkaline medium, undergoing a retrograde aldol condensation with the production of betaine and acetaldehyde. The latter compound polymerizes under the influence of alkali, giving the gummy brown precipitate which appears during the refluxing of the alkaline methylation mixture.

Under the above conditions *dl*-threonine betaine also decomposes to betaine and acetaldehyde. *dl*-Serine betaine, however, is more stable toward alkali and no evidence was obtained for the production of formaldehyde when the methylation mixture was heated. In this case the product, isolated in the usual manner, was a hygroscopic syrup which crystallized after long standing. A low yield (10 to 20 per cent) of *dl*-serine betaine hydrochloride was obtained when this material was recrystallized several times from absolute alcohol.

In an attempt to avoid these difficulties Novak's procedure was modified as follows: The methylation was carried out as before. The cold solution was then acidified with hydrochloric acid (100 cc. of 5.0 *N* per 0.1 mole of amino acid methylated) and the acid solution was refluxed for 5 hours. In this way both dimethyl sulfate and monomethyl sulfate were hydrolyzed in a single step. The betaine hydrochlorides were isolated as in Novak's procedure. In each case the product was a hygroscopic syrup which crystallized very slowly. Only a small amount of pure betaine hydrochloride could be obtained from the crude products and the purification required repeated recrystallizations. Since an excess of dimethyl sulfate is used in Novak's method, it was possible that a certain amount of O-methylation had occurred. Strong evidence supporting this view was the fact that the neutral equivalents of the partially purified products were 2 to 4 per cent higher than the calculated value. In the case of serine the O-methyl betaine hydrochloride was actually isolated in a pure state. The crude product obtained in one run was dissolved in absolute alcohol and cooled in an ice bath. An oil precipitated immediately. On further standing, large flat crystals appeared on the sides of the flask. These were carefully removed and re-



crystallized from absolute alcohol. The product thus obtained melted at 196–197°, and gave the correct analytical data for O-methyl-*dl*-serine betaine hydrochloride.

$C_7H_{16}O_3NCl$ .	Calculated.	N 7.09,	Cl 17.97,	neutral equivalent	197.5
	Found.	" 7.02,	" 17.93,	" "	200.0

This difficulty was overcome by decreasing the amount of dimethyl sulfate and by altering the conditions of the reaction slightly. Satisfactory results were finally obtained by the following procedure.

A solution of 0.1 mole of amino acid in 100 cc. of 1 *N* potassium hydroxide was placed in a 500 cc. 3-necked flask equipped with a stirrer and two small separatory funnels. 37.8 gm. (28 cc., 0.3 mole) of dimethyl sulfate were placed in one separatory funnel, and a solution of 16.8 gm. (0.3 mole) of potassium hydroxide in 28 cc. of water was placed in the other. The flask was cooled in an ice bath and the two liquids were added at the same rate to the vigorously stirred reaction mixture over a period of 30 minutes. 40 cc. of concentrated hydrochloric acid were added and the solution was refluxed for 5 hours. 75 gm. of barium chloride dihydrate in 200 cc. of water were added to the warm solution. The barium sulfate was removed by filtration and washed twice with warm water. The filtrate and washings were concentrated to a syrup *in vacuo*. The residue was extracted with 300 cc. of absolute alcohol and the insoluble material (potassium chloride) was removed by filtration. The filtrate was concentrated and the residue was dissolved in 100 cc. of absolute alcohol. The alcohol was removed under reduced pressure, giving the crude betaine hydrochloride in the form of a viscous oil. The purification of the individual products is described below.

*dl*-Serine Betaine Hydrochloride—The crude product was dissolved in 100 cc. of warm absolute alcohol. The solution was filtered, and cooled in the ice box for 48 hours. The crystalline precipitate was removed by filtration, washed with cold alcohol and ether, and dried in a vacuum desiccator. 9 gm. of *dl*-serine betaine hydrochloride were thus obtained. A small additional amount (3 gm.) of impure material precipitated from the alcoholic filtrate on the addition of ether. This was purified by recrystal-

lization from 5 volumes of hot absolute alcohol. The total yield of pure product, melting at 198–199°, was 10.8 gm. (58 per cent).

$C_6H_{14}O_3NCl$ .	Calculated.	N 7.63,	Cl 19.35,	neutral equivalent 183.5
	Found.	" 7.64,	" 19.10,	" " 185

*dl*-Serine betaine hydrochloride is slightly hygroscopic and is highly water-soluble. It is moderately soluble in ethyl and butyl alcohol and is best purified from the former solvent.

*dl*-Allothreonine Betaine Hydrochloride—The crude product was placed in a vacuum desiccator over phosphorus pentoxide for 6 days, during which time the substance slowly crystallized. The crystalline mass was dissolved in 50 cc. of hot absolute alcohol. The solution was filtered and cooled to room temperature. Anhydrous ether was added slowly until a faint haziness was produced. The solution was seeded and placed in the ice box overnight. The crystalline precipitate was removed by filtration, washed with ether, and air-dried. This material was recrystallized again from an alcohol-ether mixture, giving 9 gm. (45 per cent yield) of pure *dl*-allothreonine betaine hydrochloride melting at 166–168°. A small additional amount of product may be obtained by working up the filtrates, but it is hardly profitable to do so.

$C_7H_{16}O_3NCl$ .	Calculated.	N 7.09,	Cl 17.97,	neutral equivalent 197.5
	Found.	" 7.03,	" 17.89,	" " 198

*dl*-Threonine Betaine Hydrochloride—The crude product was purified in the same manner as the allothreonine derivative. Butyl alcohol was found to be a satisfactory solvent for recrystallization of the derivative, after it had been partially purified by one recrystallization from an alcohol-ether mixture. The yield of pure *dl*-threonine betaine hydrochloride was 8 gm. (41 per cent).

$C_7H_{16}O_3NCl$ .	Calculated.	N 7.09,	Cl 17.97,	neutral equivalent 197.5
	Found.	" 7.17	" 18.04,	" " 199

*dl*-Threonine betaine hydrochloride is considerably more soluble in absolute alcohol than the allothreonine derivative. The threonine betaine hydrochloride melts at 162–164°. A mixture of the isomers melts at 142–152°.

*Feeding Experiments*

Albino rats weighing 125 to 175 gm. were used in the feeding experiments. The rats were maintained in separate metabolism cages and fed *ad libitum*. At the end of 21 days they were decapitated and the livers removed. The total fatty acid and

TABLE I  
*Effect of Various Substances on Production of Dietary Fatty Livers in Rats*

Supplement to diet*		Daily food intake	Change in weight	Liver		
				Weight	Weight per 100 gm. rat	Lipid†
	per cent	gm.	per cent	gm.	gm.	per cent
Casein‡.....	5.0	6.1	-14.3	5.28	4.6	27.6
“.....	5.0	5.5	-12.0	6.09	6.1	27.1
“.....	10.0	7.9	16.8	7.55	4.9	31.1
“.....	20.0	8.3	26.9	7.26	4.0	14.3
“.....	30.0	7.9	37.0	6.05	3.5	9.4
“ hydrolysate (7).....	29.3	6.7	14.4	5.32	3.5	7.1
Tryptophane.....	0.7					
Casein.....	5.0	5.1	-17.9	3.79	3.4	7.5
Choline chloride.....	0.25					
Casein.....	5.0					
Betaine hydrochlorides of						
<i>dl</i> -Serine.....	1.9	5.6	-17.7	3.23	4.1	21.7
“.....	1.9	5.5	-13.5	6.90	6.2	33.4
<i>dl</i> -Threonine.....	2.0	5.5	-11.7	6.15	5.5	26.8
<i>dl</i> -Allothreonine.....	2.0	5.5	-11.2	5.76	5.1	24.9

\* Each experiment was carried out on a group of six rats (three males and three females) from the same litter.

† In these experiments, the term “lipid” is used to designate total fatty acid and unsaponifiable matter.

‡ Labco vitamin-free casein was used in all the experiments.

unsaponifiable matter of the individual fresh livers were determined by the method of Leathes and Raper (5).

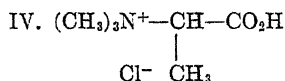
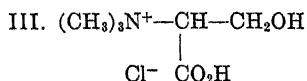
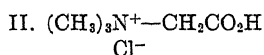
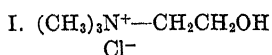
Each of the diets used contained Crisco 40, salt mixture (Osborne and Mendel (6)) 5, cod liver oil 1, the supplement, and glucose to make 100 per cent. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 10 mg. of fullers' earth adsorbate of a rice polishings

extract. The supplements, the concentrations at which they were incorporated in the diet, and the results of the feeding experiments are shown in Table I.

The results obtained with various levels of casein are similar to those reported by Channon and Wilkinson (2) and others. The basal diet (5 per cent casein) uniformly produced a highly fatty liver. A control group of rats receiving choline chloride showed no accumulation of fat in the liver.

The betaine hydrochlorides were fed at 5 times the molar level of the choline chloride. The rats showed no obvious ill effects from the ingestion of these quantities of the betaine hydrochlorides, and lost no more weight than the controls. Although there is considerable difference in the liver fat of the two groups of rats receiving serine betaine hydrochloride, the data show conclusively that the hydroxybetaines have no lipotropic effect.

Recently Welch and Welch (8) and Platt (9) have shown that choline has considerably more lipotropic effect than betaine. The former authors found that alanine betaine also has lipotropic activity but made no comparison of this substance with choline or betaine. These results combined with our data make possible a comparison of the lipotropic activity of choline chloride (I), betaine hydrochloride (II), serine betaine hydrochloride (III), and alanine betaine hydrochloride (IV). Such a comparison



affords an interesting study of variation of physiological effect with structure in a group of closely related compounds. The results show that the introduction of a hydroxyl group into the betaine molecule may depress rather than enhance its effect on liver fat. Further, it is interesting to note that the introduction of a carboxyl group on the  $\alpha$ -carbon of choline converts it into the lipotropically inactive serine betaine.

The mixture of amino acids obtained on hydrolysis of casein is as effective as casein in preventing the development of fatty livers in rats. Since the hydrolysate was practically phosphorus-

free, this result eliminates the possibility that the organic phosphorus of the casein is responsible for its lipotropic effect.

#### SUMMARY

1. A method has been developed for the synthesis of the betaine hydrochlorides of *dl*-serine, *dl*-threonine, and *dl*-allothreonine.

2. The betaines of *dl*-threonine and *dl*-allothreonine undergo a retrograde aldol condensation in an alkaline medium, yielding betaine and acetaldehyde.

3. The betaine hydrochlorides of *dl*-serine, *dl*-threonine, and *dl*-allothreonine do not prevent the development of a fatty liver in rats fed a high fat, low protein diet.

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## AZLACTONES

### II. AZLACTONE FORMATION IN GLACIAL AND IN AQUEOUS ACETIC ACID AND PREPARATION OF BENZOYL- $\alpha$ -AMINOCROTONIC ACID AZLACTONE II

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In a continuation of our work with azlactones (1) we have carried out an investigation of azlactone formation in aqueous and glacial acetic acid under the influence of acetic anhydride. Such a study was of interest in connection with the work of du Vigneaud and Meyer (2) and of Bergmann and Zervas (3) on the racemization of amino acids. The former authors found that acyl derivatives of certain amino acids in aqueous solutions were racemized by the action of acetic anhydride provided a basic substance, such as sodium acetate or pyridine, was present. In the absence of the base very little racemization occurred. Convincing evidence was advanced that azlactone formation caused the racemization, although the azlactones were not isolated from the reaction mixtures. Bergmann and Zervas (3) studied the racemization of acylated amino acids in glacial acetic acid and reported that the results obtained depended on the relative amounts of acyl derivative and acetic anhydride used in the reaction. In the presence of 2 or more moles of acetic anhydride an azlactone was formed with resulting racemization of the acyl derivative. In the presence of 1 mole or less of acetic anhydride no azlactone was isolated but "catalytic" racemization of the derivative occurred. It seemed possible that these results might be correlated by a study of the factors affecting azlactonization and racemization of acylated amino acids.

The fact that benzoyl- $\alpha$ -aminocrotonic acid azlactone is resistant to hydrolysis, and is readily isolated and recrystallized, afforded

an excellent means of studying azlactone formation. Therefore, we undertook a study of the reactions of benzoyl- $\alpha$ -aminocrotonic acid and of N-benzoyl-O-methyl-*dl*-allothreonine with acetic anhydride in aqueous and in glacial acetic acid, with and without the addition of sodium acetate. The results obtained made it desirable to study further racemization in glacial acetic acid under the conditions used by Bergmann and Zervas (3). For this purpose benzoyl-*L-p*-methoxyphenylalanine was employed.

#### EXPERIMENTAL

The benzoyl- $\alpha$ -aminocrotonic acid azlactone obtained in these experiments was found to consist of a mixture of the *cis* and *trans* isomers. In order to distinguish these, the substance previously obtained (1), melting at 95–96°, will be called Azlactone I, and the isomeric compound, melting at 144–145°, will be designated as Azlactone II. The latter compound is readily obtained by the following method.

*Benzoyl- $\alpha$ -Aminocrotonic Acid Azlactone II*—47.4 gm. of N-benzoyl-O-methyl-*dl*-allothreonine were heated on the steam cone for 10 to 15 minutes with 100 cc. of acetic anhydride. The solution was cooled in an ice bath and the precipitate was removed by filtration. The solid material was recrystallized from 8 volumes of alcohol, yielding 11 to 13 gm. (30 to 35 per cent yield<sup>1</sup>) of crude Azlactone II melting at 138–140°. The crude substance was purified by recrystallization from benzene or alcohol.

$C_{11}H_9O_2N$ .	Calculated.	C 70.59,	H 4.81,	N 7.49
	Found.	" 70.71,	" 4.96,	" 7.56

Azlactone II melts at 144–145°, if the melting point tube is placed in the bath at 135–140° and heated rapidly to the melting point. This precaution is essential, since Azlactone II, when heated at 100° or above, is rapidly converted into a mixture of the isomers melting at 83–88°. Azlactone II is converted almost quantitatively into Azlactone I by the action of pyridine at room temperature for 15 minutes. This behavior may account for the fact that in our previous work (1) only Azlactone I was obtained.

<sup>1</sup> 16 to 18 gm. of crude Azlactone I may be isolated from the acetic anhydride and alcohol filtrates from Azlactone II, giving a total yield for this reaction of 75 to 85 per cent.

The structure of Azlactone II was established by hydrolysis to Benzoyl- $\alpha$ -aminocrotonic Acid II and by conversion to benzoyl- $\alpha$ -aminobutyric acid by the method previously described.

*Benzoyl- $\alpha$ -Aminocrotonic Acid II*—10 gm. of finely powdered Azlactone II were heated on the steam cone with 800 cc. of 0.5 N hydrochloric acid for 15 minutes. The solid did not dissolve completely. The mixture was cooled in an ice bath and the precipitate was removed by filtration, yielding 7.5 gm. of crystalline material melting at 140–160°. This was extracted with 100 cc. of warm benzene, giving 6 gm. of a mixture of the benzoyl- $\alpha$ -aminocrotonic acids melting at 165–175°. The crude material was recrystallized from absolute alcohol, giving 4 gm. of pure Benzoyl- $\alpha$ -aminocrotonic Acid II.

$C_{11}H_{11}O_3N$ . Calculated. C 64.39, H 5.37, N 6.83, neutral equivalent 205  
Found. " 64.37, " 5.42, " 6.90, " " 207

Benzoyl- $\alpha$ -aminocrotonic Acid II melts at 195–198°; Acid I melts at 193–195°; a mixture of the two acids melts at 165–170°. Acid II is considerably less soluble than Acid I in absolute alcohol.

*Azlactonization of N-Benzoyl-O-Methyl-dl-Allothreonine and of Benzoyl- $\alpha$ -Aminocrotonic Acid*

*In Aqueous Acetic Acid with Sodium Acetate*—5.9 gm. (0.025 mole) of N-benzoyl-O-methyl-dl-allothreonine were suspended in 50 cc. of water containing 2.1 gm. (0.025 mole) of sodium acetate. The solution was placed in a bath at 40–45° and vigorously shaken while 30 cc. of acetic anhydride were added slowly. The benzoyl derivative rapidly dissolved and after 2 to 3 minutes a white solid began to separate. At the end of 15 minutes the solid was removed by filtration and was recrystallized from 50 per cent alcohol, giving 3.75 gm. (80 per cent yield) of a mixture of Azlactones I and II melting at 80–105°.

The above experiment was repeated with 5.1 gm. (0.025 mole) of Benzoyl- $\alpha$ -aminocrotonic Acid I. The yield of azlactone melting at 83–88° was 3.6 gm. (77 per cent).

*In Aqueous Acetic Acid without Sodium Acetate*—The above experiments were repeated without the addition of sodium acetate. The reaction was allowed to proceed for 2 hours. The yield of azlactone in each case was 0.2 to 0.3 gm. (4 to 6 per cent).



*In Glacial Acetic Acid*—0.025 mole of the benzoyl derivative was dissolved or suspended in 50 cc. of glacial acetic acid. In certain of the experiments 2.1 gm. (0.025 mole) of sodium acetate were added. Then 3 cc. of acetic anhydride (0.03 mole) were added and the mixture was maintained at a temperature of 38–40° during the course of the reaction. After varying lengths of time the solution was poured into a mixture of ice and water. The precipitate was removed by filtration, washed thoroughly with cold water, and air-dried. This material was dissolved in 10 volumes of alcohol. The solution was diluted with an equal volume of water and cooled overnight in the ice box. The pre-

TABLE I  
*Azlactone Formation in Glacial Acetic Acid*

Time of reaction	Yield of azlactone from			
	N-Benzoyl-O-methyl- <i>dl</i> -allothreonine		Benzoyl- $\alpha$ -aminocrotonic acid	
	No sodium acetate present	Sodium acetate present	No sodium acetate present	Sodium acetate present
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.0	2.1	36.2	2.1	14.9
1.5		51.1		42.6
2.0		63.9	6.4	57.5
2.5		76.6		
3.0	8.5	78.6		74.5
4.0		80.9		
6.0	17.0		12.8	87.3
24.0	63.9		68.2	

precipitate was removed by filtration and air-dried, giving a mixture of Azlactones I and II melting at 83–88°. The results of these experiments are summarized in Table I.

The following experiments were carried out to determine accurately the yield of azlactone produced by the action of 1 mole or less of acetic anhydride on 1 mole of benzoyl derivative.

23.7 gm. (0.1 mole) of N-benzoyl-O-methyl-*dl*-allothreonine were dissolved in 100 cc. of glacial acetic acid. 10.2 gm. (0.1 mole) of acetic anhydride were added and the solution was warmed on the steam cone for 15 minutes (protected from moisture by a calcium chloride tube). The solution was cooled and poured into

ice water. The precipitate was removed by filtration and recrystallized from 50 per cent alcohol, giving 15 gm. (80 per cent yield) of a mixture of Azlactones I and II melting at 83–88°.

The experiment was repeated with only 5.1 gm. (0.05 mole) of acetic anhydride. The yield of azlactone was 6 gm. (64 per cent calculated on the amount of acetic anhydride used).

*Azlactonization and Racemization of Benzoyl-l-p-Methoxyphenylalanine*

Benzoyl-*l-p*-methoxyphenylalanine was prepared by the method of Behr and Clarke (4). The product obtained had a specific rotation of  $[\alpha]_D^{28} = +9.7^\circ$  in glacial acetic acid and  $-24.4^\circ$  in 50 per cent acetic acid (2.5 per cent solutions). The large variation in rotation with change in concentration of acetic acid was of some importance in connection with the racemization studies.

Benzoyl-*dl-p*-methoxyphenylalanine azlactone was prepared by the method of Mohr and Stroschein (5). The oil remaining after concentration of the acetic anhydride was crystallized from 50 volumes of high boiling petroleum ether. The yield of pure optically inactive azlactone, melting at 80–82°, was 65 to 70 per cent of the theoretical amount.

$C_{17}H_{15}O_3N$ . Calculated, N 4.98; found, N 5.07

The azlactone is moderately soluble in petroleum ether and is quite soluble in benzene and glacial acetic acid. It is readily hydrolyzed to the benzoyl derivative by standing for a few hours in aqueous acetic acid.

Benzoyl-*dl-p*-methoxyphenylalanine anilide was prepared by warming the azlactone for 20 minutes at 60° with aniline. The pure anilide, melting at 207–209°, was obtained by recrystallizing the crude material from hot alcohol.

$C_{23}H_{22}O_3N_2$ . Calculated, N 7.48; found, N 7.49

The anilide is insoluble in water and dissolves to the extent of 1 gm. in 40 cc. of boiling alcohol.

*Azlactonization of Benzoyl-l-p-Methoxyphenylalanine*

*In Aqueous Acetic Acid*—4.0 gm. (0.013 mole) of benzoyl-*l-p*-methoxyphenylalanine, 1.1 gm. (0.013 mole) of sodium acetate,

and 13 cc. of water were placed in a 100 cc. flask. 10 cc. of acetic anhydride were added and the flask was placed in a bath at 40° and shaken intermittently for 15 minutes. The mixture was then poured into a separatory funnel containing 150 cc. of ice water and 100 cc. of benzene. The contents of the funnel were vigorously agitated for 5 minutes. The aqueous layer was removed and the benzene layer was washed with two 100 cc. portions of 10 per cent potassium bicarbonate. The benzene layer was dried over anhydrous potassium carbonate, and the benzene was removed under reduced pressure. The residue crystallized on standing in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide. The crude solid was recrystallized from 80 cc. of petroleum ether, giving 1.58 gm. (42 per cent yield) of benzoyl-*dl-p*-methoxyphenylalanine azlactone melting at 79–82°.

*In Glacial Acetic Acid*—6 gm. (0.02 mole) of benzoyl-*l-p*-methoxyphenylalanine, 20 cc. of glacial acetic acid, and 2 cc. (0.02 mole) of acetic anhydride were heated on the steam bath for 15 minutes. The reaction mixture was cooled and worked up as in the previous experiment. The yield of azlactone melting at 78–82° was 1.25 gm. (22 per cent).

In the following experiment the benzoyl derivative was treated with varying amounts of acetic anhydride, and the azlactone present in each of the reaction mixtures was estimated by conversion into the insoluble benzoyl-*dl-p*-methoxyphenylalanine anilide.

2.99 gm. (0.01 mole) of benzoyl-*l-p*-methoxyphenylalanine and 10 cc. of glacial acetic acid were placed in each of four flasks. Acetic anhydride was added to each flask in different amounts. The flasks were heated on the steam cone for 15 minutes and 10 cc. of aniline were added to each flask. After 3 minutes 100 cc. of water were added and the flasks were kept in the ice box overnight. The precipitates were removed by filtration and recrystallized from 40 volumes of hot alcohol. Pure benzoyl-*dl-p*-methoxyphenylalanine anilide was thus obtained in yields of 0.80, 1.38, 1.79, and 2.24 gm. respectively from 0.01, 0.02, 0.03, and 0.05 mole of acetic anhydride. 2.81 gm. (0.01 mole) of pure azlactone dissolved in glacial acetic acid and treated with aniline gave 2.5 to 2.7 gm. of anilide.

*Reaction of Azlactones with Acetic Acid*

The difference in yield of anilide with increasing amounts of acetic anhydride indicates that azlactone formation involves a reversible reaction whose equilibrium point is greatly influenced by the amount of acetic anhydride present. In order to obtain further information on this point the reaction of azlactones with acetic acid was investigated.

A solution of 2.81 gm. (0.01 mole) of benzoyl-*dl*-*p*-methoxyphenylalanine azlactone in 10 cc. of glacial acetic acid was heated on the steam cone for 15 minutes. 5 cc. of aniline were added and the solution worked up as in the previous experiment. The yield of recrystallized anilide melting at 205–207° was only 1.2 gm., as compared to a yield of 2.5 gm. with unheated azlactone. The original filtrate was neutralized with solid sodium hydroxide and extracted with ether. The ether extract was washed with a small amount of dilute hydrochloric acid, dried, and evaporated. The residue was recrystallized from water, giving a small amount of pure acetanilide melting at 111–113°.

A solution of 5 gm. of benzoyl-*dl*-phenylalanine azlactone (5) in 10 cc. of glacial acetic acid was heated at 90–100° for 2 hours and was then placed in the ice box overnight. The precipitate was removed by filtration and air-dried, giving 2.84 gm. of benzoyl-*dl*-phenylalanine melting at 176–182° (6). The filtrate was distilled under reduced pressure into a flask containing 2 cc. of aniline. The receiving flask was then warmed on the steam cone for 5 minutes. The solution was cooled and taken up in ether. The ether solution was washed with cold concentrated sodium hydroxide and with cold hydrochloric acid. The ether was evaporated and the residue was recrystallized from water, giving 0.43 gm. of acetanilide melting at 112–113°. The residue in the distillation flask was extracted with hot petroleum ether and the extract was concentrated to a small volume and cooled in the ice box, yielding 0.5 gm. of crude benzoyl-*dl*-phenylalanine azlactone.

Similar results were obtained when the above experiment was repeated with benzoyl-*dl*-*p*-methoxyphenylalanine azlactone.

*Racemization of Benzoyl-l-p-Methoxyphenylalanine*

*By Acetic Anhydride*—Benzoyl-*l*-*p*-methoxyphenylalanine was rapidly racemized by the method of Bergmann and Zervas (3),

with amounts of acetic anhydride varying from 0.5 to 3.0 moles per mole of benzoyl derivative. It was also racemized readily by the method of du Vigneaud and Meyer (2). The benzoyl-*dl-p*-methoxyphenylalanine thus obtained melted at 173–174°.² The racemic benzoyl derivative is very slightly soluble in glacial acetic acid, while the optically active isomer is readily soluble in both glacial acetic acid and 50 per cent acetic acid.

*By Benzoyl-dl-p-Methoxyphenylalanine Azlactone*—It was discovered, rather unexpectedly, that an azlactone will cause rapid racemization of the benzoyl derivative. As a preliminary experiment 0.84 gm. (0.003 mole) of azlactone was added to a solution of 9.0 gm. of benzoyl derivative in glacial acetic acid at room temperature. In a short time the racemic derivative began to separate. At the end of 12 hours the precipitate was removed, yielding 2.7 gm. (0.009 mole) of benzoyl-*dl-p*-methoxyphenylalanine melting at 173–175°.

In order to study this reaction further, quantitative determinations were made of the rates of racemization of benzoyl-*l-p*-methoxyphenylalanine by azlactone and by acetic anhydride in the presence and in the absence of sodium acetate. These experiments were carried out in glacial acetic acid at room temperature, under which conditions the racemization proceeded at a readily measurable rate. In preliminary runs the acetic acid solution of the reactants was placed in a polarimeter tube and the rotation was determined at intervals. However, this method was limited by precipitation of the racemic derivative. The following procedure was finally adopted in order to take advantage of the high rotation of benzoyl-*l-p*-methoxyphenylalanine in 50 per cent acetic acid.

2.99 gm. (0.01 mole) of the benzoyl derivative were dissolved in 45 cc. of glacial acetic acid. In certain experiments 1.02 gm. (0.01 mole) of sodium acetate were added. The racemizing agent was added and the final volume was brought to 50 cc. with acetic acid. 5 cc. samples were removed at intervals, the first immediately after the reactants were thoroughly mixed. The samples were added to 5 cc. of 0.25 N hydrochloric acid. In those experi-

² Dakin (7) found this compound to melt at 135–136°. Later, Lamb and Robson (8) reported a melting point of 173° but did not refer to Dakin's work.

ments in which no sodium acetate was present in the acetic acid solution, 0.1 gm. was added to the hydrochloric acid. The mixtures were allowed to stand for 12 hours, the solutions were filtered, and the rotations were determined. Control experiments showed that no racemization of the derivative occurred during the 12 hours standing in the 50 per cent acetic acid solution

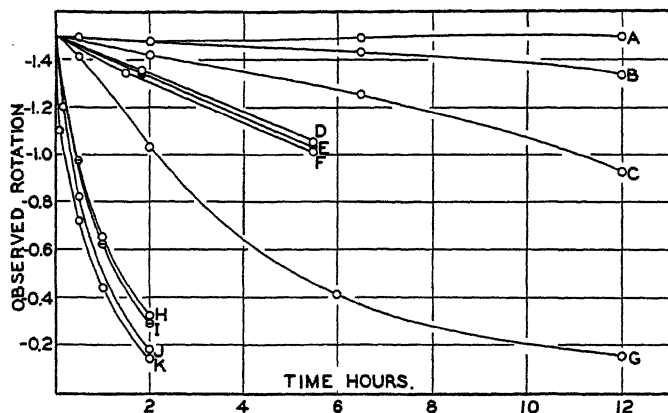


FIG. 1. Racemization of benzoyl-*l*-*p*-methoxyphenylalanine (0.01 mole) in glacial acetic acid by the action of: Experiment A, benzoyl- $\alpha$ -amino-crotonic acid azlactone (0.01 mole); Experiment B, acetic anhydride (0.01 mole); Experiment C, acetic anhydride (0.03 mole); Experiments D, E, F, conditions described in the text; Experiment G, acetic anhydride (0.01 mole) and sodium acetate (0.01 mole); Experiment H, benzoyl-*dl*-alanine azlactone (5) (0.01 mole); Experiment I, benzoyl-*dl*-phenylalanine azlactone (0.01 mole); Experiment J, benzoyl-*dl*-*p*-methoxyphenylalanine azlactone (0.01 mole); Experiment K, benzoyl-*dl*-*p*-methoxyphenylalanine azlactone (0.01 mole) and sodium acetate (0.01 mole).

containing the racemizing agent and a small amount of sodium chloride and hydrochloric acid.

Certain alterations were made in the procedure in Experiments D, E, and F. In Experiments D and E a solution of 0.01 mole of acetic anhydride and 0.01 mole of benzoyl-*dl*-*p*-methoxyphenylalanine in 50 cc. of glacial acetic acid was heated for 2 hours at 90–100°. In Experiment F a solution of 0.01 mole of benzoyl-*dl*-*p*-methoxyphenylalanine azlactone in 50 cc. of glacial acetic acid was heated similarly. 0.01 mole of benzoyl-*l*-*p*-methoxy-

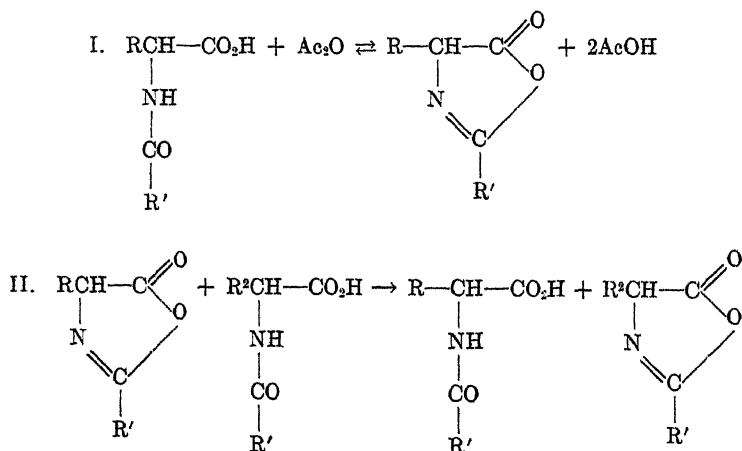
phenylalanine was added to each of the three solutions and the rotations were followed in the usual manner.

The results of these experiments are shown in Fig. 1.

#### DISCUSSION

These experiments indicate that the racemization of an acylated amino acid by excess acetic anhydride, in either aqueous or glacial acetic acid, occurs by the same mechanism and depends in each case on the formation of an azlactone as the racemizing intermediate. Sodium acetate increases the rate of azlactonization and rate of racemization in both glacial and aqueous acetic acid. It seems probable, therefore, that the main rôle of the sodium acetate consists, not in accelerating racemization of the azlactone, but in increasing the rate of azlactonization. In fact, in aqueous acetic acid the acetic anhydride reacts so rapidly with water that little azlactone is produced unless the rate of reaction of acetic anhydride with the acyl derivative is increased by the addition of sodium acetate.

The racemization of an acylated amino acid by less than 1 mole of acetic anhydride may be explained by either of the reactions shown in Equations I and II.



Equation I has been postulated by Clarke (9) as a possible explanation of Bergmann's "catalytic" racemization. The exist-

ence of such an equilibrium seems to be well established by our results. However, Equation I does not satisfactorily explain the racemization of benzoyl-*l-p*-methoxyphenylalanine by the various azlactones, since this racemization is much more rapid than that produced by an equivalent amount of acetic anhydride. It is very probable that this effect involves a chemical reaction between the azlactone and the benzoyl derivative, since the unreactive benzoyl- $\alpha$ -aminocrotonic acid azlactone causes no racemization of benzoyl-*l-p*-methoxyphenylalanine. The rapidity with which the racemization occurs suggests that an azlactone may be the racemizing intermediate, as shown in Equation II. This reaction (or, perhaps, a combination of the two reactions) seems to afford the most logical explanation of the "catalytic" racemization.

In continuing this work we have discovered that certain acyl derivatives of *l*-proline and of N-methyl-*d*-phenylalanine are rapidly racemized by the action of acetic anhydride in glacial acetic acid. Since azlactonization is impossible in these compounds, some other intermediate is responsible for the racemization. The nature of the intermediate is being investigated, and a study is being made of the effect of variation in the acyl group on the rate of racemization of acylated amino acids in glacial and aqueous acetic acid.

#### SUMMARY

1. A study has been made of the azlactonization of benzoyl- $\alpha$ -aminocrotonic acid and of N-benzoyl-O-methyl-*dl*-allothreonine in aqueous and in glacial acetic acid. Both the *cis* and *trans* isomers of benzoyl- $\alpha$ -aminocrotonic acid azlactone are produced in certain of these reactions.

2. The azlactonization and racemization of benzoyl-*l-p*-methoxyphenylalanine have been investigated.

3. The azlactones of benzoyl-*dl-p*-methoxyphenylalanine, benzoyl-*dl*-phenylalanine, and benzoyl-*dl*-alanine cause a rapid racemization of benzoyl-*l-p*-methoxyphenylalanine in glacial acetic acid.

4. Racemization of acylated amino acids by the procedure of du Vigneaud and Meyer (2) and of Bergmann and Zervas (3) is discussed in the light of these studies.



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## EFFECT OF REPEATED INSULIN HYPOGLYCEMIA ON THE LIPID COMPOSITION OF RABBIT TISSUES

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The wide usage of large hypoglycemic doses of insulin as a therapeutic agent in the treatment of psychoses prompted the study of the effects of such treatment on the lipid composition of tissues, particularly of the brain, in experimental animals. Although it is generally recognized that the brain utilizes only carbohydrate for energy under normal conditions, while other tissues have the ability to oxidize fat, it is not known what the brain uses for energy during prolonged hypoglycemia when the carbohydrate reserves are exhausted. On the basis of a wide-spread destruction of brain cells which was accompanied by a movement of water and potassium out of the cells, Yannet (1) postulated that some of the brain cells die when the carbohydrate reserves of nervous tissue are exhausted during prolonged insulin hypoglycemia.

It has been conclusively demonstrated that the metabolism of phospholipid in the brain is an extremely slow process (2), indicating that phospholipid has a structural rather than a metabolic significance. Under normal conditions phospholipid and probably cholesterol are apparently replaced only as the result of the process of wear and tear of the tissue. We wished to study the effects of abnormal metabolism such as must occur under insulin hypoglycemia on the lipid composition of the brain. We have included the adrenal glands, which are known to hypertrophy under the influence of insulin (3). We were further interested in possible changes in the liver, kidney, spleen, and muscle.

### *Methods*

The control and experimental rabbits matched for age and weight were kept for similar periods of time on the same vege-

table diet. The experimental animals were given a convulsive dose (3 to 10 units per kilo) daily. After an animal had several severe convulsions, it was given 25 cc. of 50 per cent sugar solution by stomach tube. During the period of treatment, the animals received 300 to 600 units of insulin and had fifteen to thirty convulsions. All of the experimental animals died in convulsion and the controls were killed with ether.

Weighed samples of the various tissues were extracted repeatedly with boiling alcohol and ether. Duplicate aliquots of the extract were used for lipid determinations. Phospholipid was determined by Bloor's method (4). Acetone-soluble lipid was oxidized directly after evaporation of the solvent and calculated according to Bloor's method for total fatty acid plus cholesterol (5). Free cholesterol was precipitated as the digitonide from the acetone-soluble fraction in a mixture of 1 cc. of 1 per cent digitonin in alcohol, 1 cc. of alcohol, 2 cc. of acetone, 2 cc. of water, and 3 drops of 5 per cent HCl. After standing for 24 hours, the digitonide was centrifuged, washed with hot water, acetone, and ether, transferred to oxidation flasks, and oxidized by the Okey procedure (6). In the case of the adrenal glands, total cholesterol was saponified in centrifuge tubes according to the method of Sperry and Stoyanoff (7). After acidification with HCl, the procedure followed that for free cholesterol. Neutral fat in the adrenal glands was calculated as total acetone-soluble fat minus total cholesterol minus 67 per cent of cholesterol esters. Total lipid was taken as the sum of phospholipid and acetone-soluble lipid.

Fisher's *t* value (8) was used for the statistical appraisal of the data.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{N_1 + N_2}{N_1 N_2 (N_1 + N_2 - 2)} \left[ SX_1^2 - \frac{(SX_1)^2}{N_1} + SX_2^2 - \frac{(SX_2)^2}{N_2} \right]}}$$

If the probability attaching to *t* was not greater than 0.05, we designated the difference as significant and, if 0.01 or less, as highly significant.

### Results

The mean values together with the pertinent statistical criteria for the lipid fractions of the various tissues of the seventeen

control and seventeen insulin-treated rabbits are recorded in Tables I and II. From Table I it may be observed that phospholipid in the insulin-treated animals is significantly lower in the nervous tissues, with the exception of the midbrain, than in the controls, while it is not different in the other tissues.<sup>1</sup> No significant differences in the mean cholesterol values were observed in any of the tissues. The acetone-soluble lipid, which includes cholesterol and neutral fat, is significantly lower in the whole brain, hemisphere, and pons; it is not different in the midbrain, cervical and thoracic cord, spleen, and muscle; and it is higher in the liver and kidney of the insulin-treated than in the control animals. Since the free cholesterol does not show significant differences between control and experimental animals and cholesterol esters are usually present in relatively unimportant amounts, it is considered that the changes in the acetone-soluble lipid fraction are due to variations in neutral fat. Therefore neutral fat must be decreased in the brain and increased in the liver and kidney of the experimental animals. The mean values for total lipid in the treated animals are significantly lower in the nervous tissues, with the possible exception of the midbrain; they are not different in the liver, muscle, and spleen; and they are higher in the kidney.

The statistically significant differences between the mean values of the various lipid fractions of insulin-treated and control animals are admittedly small. However, on the assumption that the samples are random, a real difference is indicated when the probabilities attaching to the differences are less than one in a hundred. Furthermore, the consistency of the differences in mean values in the various nervous tissues indicates that real differences have been demonstrated.

From Table II it may be observed that when calculated on the basis of moist weight cholesterol esters are significantly lower, while neutral fat is significantly higher in the adrenals in the insulin-treated than in the control animals. The mean values for phospholipid, free cholesterol, and total lipid are not significantly different. The changes in cholesterol esters are emphasized further by the significant rise in the phospholipid-total cholesterol ratio and the ester cholesterol-free cholesterol ratio.

<sup>1</sup> The values on whole brain were calculated from the weights and percentage composition of the parts; *i.e.*, hemisphere, midbrain, and pons.

TABLE I  
*Mean Values in Per Cent of Moist Weight and Statistical Criteria of Tissue Lipids in Seventeen Control and  
 Seventeen Insulin-Treated Rabbits*

Tissue	Phospholipid				Cholesterol				Acetone-soluble lipid				Total lipid				
	Mean	s.d.	t	P	Mean	s.d.	t	P	Mean	s.d.	t	P	Mean	s.d.	t	P	
Whole brain	C.	6.72	0.30	1.50	>0.05	1.93	0.17	0.36	>0.05	2.68	0.25	2.75	<0.01	9.40	0.44	2.43	<0.05>0.01
	I.	6.51	0.48			1.95	0.15			2.48	0.17			8.99	0.56		
Hemisphere	C.	5.81	0.24	2.50	<0.02>0.01	1.62	0.11	0.26	>0.05	2.21	0.18	2.88	<0.01	8.02	0.35	3.62	<0.01
	I.	5.53	0.39			1.63	0.12			2.04	0.16			7.57	0.39		
Midbrain	C.	7.91	0.59	1.71	>0.05	2.34	0.21	0.77	>0.05	3.16	0.24	1.42	>0.05	11.07	0.73	1.68	>0.05
	I.	7.58	0.54			2.28	0.24			3.04	0.25			10.62	0.73		
Pons	C.	10.68	0.67	2.56	<0.02>0.01	3.39	0.31	0.98	>0.05	4.62	0.36	3.95	<0.01	15.30	0.71	4.07	<0.01
	I.	10.06	0.74			3.28	0.35			4.15	0.33			14.21	0.93		
Cervical cord	C.	13.90	0.93	3.53	<0.01	4.55	0.27	1.59	>0.05	5.36	0.50	0.56	>0.05	19.26	1.03	2.39	<0.05>0.01
	I.	12.80	0.78			4.34	0.41			5.25	0.56			18.05	1.19		
Thoracic cord	C.	15.44	0.81	3.11	<0.01	5.09	0.23	1.70	>0.05	6.20	0.62	0.60	>0.05	21.64	1.21	2.64	<0.05>0.01
	I.	14.39	0.99			4.78	0.63			6.06	0.64			20.45	1.45		
Liver	C.	2.62	0.41	1.96	>0.05	0.21	0.04	0.46	>0.05	1.09	0.22	3.33	<0.01	3.71	0.50	0.47	>0.05
	I.	2.29	0.56			0.20	0.04			1.50	0.46			3.79	0.71		
Spleen	C.	1.62	0.12	0.40	>0.05	0.34	0.07	0.52	>0.05	0.89	0.30	0.00	>0.05	2.51	0.35	0.25	>0.05
	I.	1.64	0.17			0.33	0.05			0.89	0.30			2.53	0.35		
Kidney	C.	2.50	0.18	1.79	>0.05	0.37	0.06	1.25	>0.05	1.86	0.51	3.33	<0.01	4.36	0.51	2.08	<0.05>0.01
	I.	2.33	0.34			0.39	0.05			2.48	0.52			4.81	0.71		
Muscle	C.	0.70	0.09	1.43	>0.05	0.061	0.07	0.00	>0.05	0.52	0.23	1.41	>0.05	1.22	0.25	1.74	>0.05
	I.	0.74	0.08			0.061	0.08			0.64	0.26			1.38	0.25		

C. = control; I. = insulin-treated.

TABLE II

*Mean Values and Statistical Criteria of Lipids in Adrenals of Seventeen Control and Seventeen Insulin-Treated Rabbits*

Phospholipid		Cholesterol			Neutral fat	Total lipid	Phospholipid Cholesterol	Ester cholesterol Free cholesterol	Adrenal weight, mg.	
		Total	Free	Ester						
In per cent of moist adrenal weight										
C.	Mean	3.57	6.30	0.80	5.50	5.82	19.26	0.59	7.09	213
	s.d.	0.33	1.42	0.22	1.22	2.93	4.46	0.15	1.02	48
I.	Mean	3.64	4.09	0.69	3.40	10.35	20.35	0.95	5.33	355
	s.d.	0.45	0.97	0.23	0.86	4.38	3.70	0.28	1.77	86
t		0.52	5.42	1.51	5.95	3.74	0.80	5.00	5.60	6.36
P		>0.05	<0.01	>0.05	<0.01	<0.01	>0.05	<0.01	<0.01	<0.01
In mg. per kilo body weight										
C.	Mean	3.52	5.88	0.75	5.18	5.69	18.46			94
	s.d.	1.21	2.27	0.30	1.92	3.81	7.73			28
I.	Mean	5.43	6.20	0.96	5.14	16.34	32.04			150
	s.d.	1.64	1.99	0.41	1.59	9.10	12.90			37
t		4.06	0.45	1.79	0.07	4.78	3.95			
P		<0.01	>0.05	>0.05	>0.05	<0.01	<0.01			

C. = control; I. = insulin-treated.

TABLE III

*Statistical Analysis of Data from Page et Al. (9) Mean Values, in Per Cent of Moist Weight, for Lipids of Eleven Normal and Ten Insulin-Treated Rabbits*

		Phospholipid			Cholesterol			Total lipid		
		Mean	s.d.	t	Mean	s.d.	t	Mean	s.d.	t
Brain	C.	6.05	0.42	1.86*	2.02	0.30	0.98*	7.73	0.75	1.05*
	I.	6.36	0.35		2.33	0.23		7.34	0.94	
Liver	C.	2.90	0.45	0.46*	0.33	0.67	0.67*	3.28	0.43	0.67*
	I.	2.98	0.37		0.32	0.05		3.17	0.26	
Kidney	C.	2.65	0.18	1.16*	0.45	0.13	2.85†	3.46	0.34	0.07*
	I.	2.57	0.13		0.58	0.08		3.44	0.54	

C. = control; I. = insulin-treated.

\*  $P = >0.05$ .

†  $P = >0.01 < 0.05$ .

Since there is a significant increase in the adrenal weights in the experimental animals, it is desirable to calculate the results on the basis of mg. per kilo of body weight, which eliminates the variation in percentage composition due to variation in organ weight. It is apparent that the results are decidedly different. Whereas phospholipid is now significantly higher in the adrenals of treated animals, total and ester cholesterol are not different, while the differences in neutral fat and total lipid are exaggerated.

#### DISCUSSION

In Table III is presented a statistical analysis of data from Page *et al.* (9). It includes the means and statistical criteria of the lipids of organs obtained from eleven normal and ten insulin-treated rabbits. The treated animals were given a single fatal dose of insulin. It is apparent that the single dose of insulin had no significant effect on the lipids of the brain and liver, while in the kidney only the cholesterol is apparently increased.

Our results indicate that after repeated insulin convulsions the brain and spinal cord lose phospholipid and perhaps neutral fat, while cholesterol remains unchanged. From the fact that a single convulsive attack is unable to affect brain phospholipid significantly it appears unlikely that the brain is able to use this fat for energy metabolism. The histological evidence, reviewed by Weil, Liebert, and Heilbrunn (10), indicates an extensive destruction of brain cells in repeated hypoglycemic treatment. The disintegration of the nerve fibers following the death of the nerve cells probably accounts for the loss of phospholipid in nervous tissue. The split-products of the phospholipid and the neutral fat are likely carried away by the blood stream. It is difficult to account for the fact that cholesterol does not disappear at the same time. In degenerating peripheral nerves, it was found that phospholipid and cholesterol disappeared at the same rate (11), while neutral fat increased. It is possible that the central nervous system has a mechanism for removing fatty acids as neutral fat but is unable to remove cholesterol.

There exists considerable doubt concerning the changes which insulin, in single injections, may produce on the fat content of skeletal muscles and internal organs. According to Theis (12)

the injection of a single lethal dose of insulin produces a decrease in the phospholipid and a proportionate increase in the neutral fat content of the liver; this is denied by MacLachlan (13) and Page *et al.* (9). Roper and Smith (14) observed a slight rise in liver fat, while Omura and Nitta (15) observed a fall. In addition, the former authors observed a rise in muscle fat; the latter also found a rise in muscle fat as well as an increase in heart and kidney fat. Page *et al.* (9) found no changes in the lipids of the heart and only a possible increase in kidney cholesterol.

In chronic hypoglycemic treatments over a period of days Theis (12) observed an increase in neutral fat and a decrease in phospholipid, similar to that which was found in acute experiments on liver fat. Our results confirm this shift in so far as the change in neutral fat content of the liver is significant but the slight lowering in the mean values for phospholipid is probably insignificant. Our results further indicate a rise in neutral fat content of the kidney but no changes in spleen or muscle.

So far as the author is aware, the effect of insulin on the lipid content of the adrenal glands has not heretofore been studied. In 1924, Riddle *et al.* (3) observed that insulin convulsive treatments produced an hypertrophy of the adrenal glands, a finding which we have confirmed. In the hypertrophied organ we have found a decrease in the cholesterol esters, when calculated as per cent of moist weight, while the absolute amount of cholesterol esters remains unchanged. The content of free cholesterol remains unchanged in either case. These results indicate that cholesterol and its esters are not concerned with adrenal hypertrophy. However, Anderson and Sperry (16) found that cholesterol esters were decreased in both percentage and absolute amounts in the hypertrophied organs of spayed, pregnant, and parturient rats.

The increase in the absolute amounts of phospholipid in the hypertrophied organs indicates that this substance is concerned with the hypertrophying process. It is probable that as the adrenals hypertrophy phospholipid is synthesized *in situ* in the newly formed cells. The increased content of neutral fat is probably part of the mechanism for providing the fatty acids essential for phospholipid synthesis.



## SUMMARY

Repeated insulin hypoglycemia convulsions were induced in seventeen rabbits. Seventeen untreated rabbits served as controls. The brain, liver, kidney, spleen muscle, and adrenals were analyzed for lipids. The insulin treatments produced a small but statistically significant decrease in the phospholipid and neutral fat content of nervous tissues but no change in cholesterol.

Phospholipid and cholesterol were not significantly affected in the liver, kidney, spleen, and muscle. Neutral fat was increased significantly in the liver and kidney only.

The adrenal glands were hypertrophied. In these glands the absolute amounts of phospholipid and neutral fat were increased, while free and ester cholesterol remained constant. On a percentage basis the amounts of phospholipid and free cholesterol remained constant; the ester cholesterol was decreased and neutral fat increased.

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## EFFECT OF TESTOSTERONE ON SERUM LIPIDS IN SCHIZOPHRENIA

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The relationship of male sex hormones to lipid metabolism has not been conclusively demonstrated. Whereas Blinoff (1) found no significant changes in the blood cholesterol values of dogs before and after castration, Kochakian *et al.* (2) found that the plasma lipids paralleled the nutritive state of castrates; fat castrates had high values, while thin castrates had normal values.

McCullagh, McCullagh, and Hicken (3) observed a common tendency to a fall in the level of blood cholesterol during a period of injection of androgenic urinary extracts into hypogonadal and eunuchoid subjects. This tendency was more pronounced in fat eunuchs who had extremely high blood cholesterol values. However, Kochakian *et al.* (2) state that no noteworthy change occurred in the plasma lipids of a normal dog, a thin castrate, or a fat castrate following the daily injection of as much as 60 mg. of androstenedione for 3 days. Likewise, in two castrate dogs testosterone oxime and testosterone benzoate, injected for a period of 2 days, had no significant immediate or delayed effect on plasma lipids.

As a part of a larger program on the therapeutic use of the male sex hormone, testosterone propionate, and its effects on many physiological variables on schizophrenic subjects, we have studied the effects of repeated injection of testosterone in oil on the serum lipids of patients. As control studies the variation in serum lipids of a series of patients treated with comparable amounts of sesame oil were followed. The studies of the effects of testosterone on normal subjects are being carried out and will be presented in a subsequent paper.

*Methods*

25 mg. of testosterone propionate, dissolved in 1 cc. of sesame oil, were injected three times a week for a period of 3 weeks into a series of nine schizophrenic subjects. Similar amounts of sesame oil were injected into another series of ten schizophrenic subjects.

Blood samples were taken on the testosterone-treated subjects at weekly intervals for 2 weeks preceding treatment, for 3 weeks during treatment, and for 1 week following treatment. In the oil control series, two samples were taken before and two during treatment at weekly intervals. The serum lipids were analyzed by methods previously described (4).

*Results*

The effects on serum lipids of 3 weeks treatment of nine schizophrenic subjects with testosterone propionate are presented in Table I. The means presented show a statistically significant upward shift in the various lipid fractions during the period of treatment, followed by a return toward the initial level after treatment.<sup>1</sup> Although there is considerable individual variation, the trend in lipid values is usually upward during the period of treatment, with the maximum in the 3rd week. The trend is usually downward toward the initial level in the 1st week following treatment.

Table II contains the means and standard errors of the lipids of the ten schizophrenic subjects treated with sesame oil. It is readily apparent that there is no significant shift in any of the lipid fractions. Thus the oil menstruum could not account for the changes noted in the testosterone-treated series.

<sup>1</sup> The data were tested by Fisher's analysis of variance. The  $F$  ratios were formed from

$$\frac{\text{Between means of period variance}}{\text{Interaction variance}}$$

where the variance between means of periods measures the scatter not of single readings but of the average reading of periods around the group mean, and the interaction variance measures the variation not ascribable to the individuals or to the periods. The  $P$  values corresponding to the  $F$  ratios were taken from Snedecor's tables. If  $P_F$  is less than 0.05, we call the means significantly different; if  $P_F$  is less than 0.01, this difference between the period means is highly significant.

TABLE I

*Means and Standard Errors of Serum Lipids of Nine Schizophrenic Subjects Treated with Testosterone*

The mean values are given in mg. per cent. The  $\pm$  figure represents the standard error =  $s/\sqrt{n-1}$  where  $s = \sqrt{(\sum(x - \bar{x})^2)/(n-1)}$ .

$n = 9$	Premedication		Medication			Postmed-ication
	1st wk.	2nd wk.	1st wk.	2nd wk.	3rd wk.	1st wk.
	Mean	Mean	Mean	Mean	Mean	Mean
Phospholipid*	186 $\pm 8.25$	184 $\pm 7.36$	202 $\pm 9.93$	203 $\pm 7.57$	224 $\pm 8.29$	186 $\pm 4.04$
Total cholesterol*	172 $\pm 5.86$	174 $\pm 4.86$	186 $\pm 8.68$	188 $\pm 7.89$	198 $\pm 6.00$	169 $\pm 7.25$
Free cholesterol*	50 $\pm 1.71$	52 $\pm 1.61$	54 $\pm 3.21$	54 $\pm 1.96$	56 $\pm 1.96$	48 $\pm 2.18$
Ester cholesterol*	122 $\pm 4.57$	122 $\pm 3.89$	132 $\pm 5.93$	134 $\pm 6.04$	141 $\pm 3.61$	121 $\pm 5.29$
Neutral fat†	113 $\pm 10.71$	108 $\pm 13.50$	132 $\pm 12.86$	137 $\pm 9.57$	151 $\pm 12.04$	106 $\pm 13.07$
Total lipid*	555 $\pm 19.79$	548 $\pm 21.46$	608 $\pm 30.32$	620 $\pm 25.64$	658 $\pm 20.64$	529 $\pm 19.11$

All trends of the above values are significant ( $< 0.01$ ) except for neutral fat which falls between 0.05 and 0.01 (still significant).

\*  $P_F < 0.01$ .

†  $P_F < 0.05 > 0.01$ .

TABLE II

*Means and Standard Errors of Serum Lipids in Ten Schizophrenic Subjects Treated with Sesame Oil*

The mean values are given in mg. per cent.

$n = 10$	Premedication				Medication			
	1st wk.		2nd wk.		1st wk.		2nd wk.	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Phospholipid.....	193 $\pm$ 4.33		192 $\pm$ 3.87		193 $\pm$ 3.27		193 $\pm$ 3.70	
Total cholesterol..	176 $\pm$ 6.07		176 $\pm$ 5.60		174 $\pm$ 5.80		175 $\pm$ 5.57	
Free " ..	50 $\pm$ 1.63		52 $\pm$ 1.90		49 $\pm$ 2.10		49 $\pm$ 1.97	
Ester " ..	126 $\pm$ 4.97		124 $\pm$ 4.10		125 $\pm$ 4.23		125 $\pm$ 3.93	
Neutral fat.....	118 $\pm$ 9.20		120 $\pm$ 10.43		116 $\pm$ 10.77		105 $\pm$ 10.73	
Total lipid.....	570 $\pm$ 16.60		572 $\pm$ 18.27		567 $\pm$ 16.77		555 $\pm$ 18.60	

None of the above trends is significant; all give  $P_F > 0.05$ .

## DISCUSSION

The continuous rise in the serum lipid values in the schizophrenic subjects during the 3 weeks of treatment together with the absence of change in sesame oil-treated subjects indicates some real effect of testosterone in the patients. However, the results of Kochakian *et al.* (2) on normal and castrate dogs indicate that a short period of treatment with male sex hormone has no effect on blood lipids. Previous results (4) on schizophrenic subjects, pointing to a low lipid level and a return toward the normal level during prolonged insulin treatment, were considered to afford evidence of an abnormal lipid metabolism in the schizophrenic subject. The present results with testosterone treatment indicate that the sex hormones may play a part in the maintenance of a normal lipid metabolism. The fact that a certain proportion of castrated animals becomes fat, while others remain thin, indicates that fat metabolism has been disturbed in some of the animals at least. Furthermore, sex hormones are apparently necessary for the maintenance of the normal weight and histological structure of the adrenal, pituitary, and thyroid glands (5). It is possible that the male sex hormone raises the abnormally low lipid values in the schizophrenic subjects by an indirect action on other glands of internal secretion.

## SUMMARY

The injection of testosterone propionate during a period of 3 weeks into nine schizophrenic subjects produced a continuous rise in serum lipids followed by a return toward the initial level after medication. Sesame oil injections had no significant effect in ten subjects.

We acknowledge our indebtedness to the Schering Corporation for the testosterone propionate used; this was supplied through Dr. R. G. Hoskins.

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## THE ACTION OF PAPAIN AND TRYPSIN ON CERTAIN DEHYDROGENASES

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Tissue suspensions of dehydrogenases often rapidly lose their activity. This loss may be caused by the action of proteolytic enzymes. To test this possibility the effect of trypsin and papain on the dehydrogenases was studied. Tissue preparations containing several dehydrogenases and very little proteinase were used. The results showed that the added proteinases attacked some dehydrogenases much more rapidly than others.

### EXPERIMENTAL

The liver and kidney of the rat were used as the source of the dehydrogenases. 10 gm. of liver or 3 gm. of kidney were chopped with scissors, ground with sand and water, and squeezed through muslin. The suspensions were then adjusted to pH 6.6 by the addition of a small amount of acetic acid, made up to 50 cc. with water, and centrifuged. The liquid was discarded and the solid was suspended in another 50 cc. of water and acidified and centrifuged again. This was done twice for the kidney and three times for the liver. In both cases the liquid after final centrifugation contained only traces of hemoglobin. Further washings had no effect on the results, so that changes in the oxidation rate of *p*-phenylenediamine, which was used to measure the activity of the cytochrome-cytochrome oxidase system, were not due to changes in the hemoglobin, which according to Elliott and Meigs (1) can also oxidize the amine. After the final washing the solid was suspended in 0.05 M phosphate buffer at either pH 6.7 for the action of papain or pH 7.6 for the action of trypsin. This amount of phosphate buffer did not interfere with the formol

titration which was carried out at intervals concurrently with testing the activity of the dehydrogenases in the Warburg apparatus. The tissue preparations alone showed no oxygen uptake.

The papain was a preparation from Eimer and Amend labeled papayotin and the trypsin was pancreatin from Merck which also contained amylase and lipase. The enzymes were added to the tissue preparations, so that the final concentration was 1.0 mg. per cc. Immediately after mixing an aliquot was taken for the formol titration and for measuring the oxidation rate. The amount of inactivation was proportional to the amount of hydrolysis.

The preparations with and without papain or trypsin were incubated at 40° in flasks open to the air. No preservative was necessary and the papain was used without cyanide. Inactivation of the dehydrogenases was complete in 50 to 100 minutes, depending on the relative concentrations of tissue and proteinase. The control kept its initial activity for 250 to 350 minutes with the exception that the oxidation rate of *p*-phenylenediamine fell off gradually after 100 minutes. The final disappearance of activity in the control was not due to oxidation of —SH groups.

In the liver, the succinoxidase, cytochrome-cytochrome oxidase system, the choline oxidase, and the amine oxidase were studied; in the kidney, only the first two and the *d*-amino acid oxidase. Experiments on the succinoxidase and cytochrome system in heart and brain showed exactly similar results. 0.5 cc. of the tissue preparation after incubation for various times with and without the proteinases was added to 1.3 cc. of buffer, pH 7.8, in the Warburg vessels and after temperature equilibration 0.2 cc. of the substrate containing 2.0 mg. was added. Readings were then taken 10, 20, and 30 minutes after the addition of the substrate. The 20 minute readings of the control are designated 100 per cent, and in Table I the 20 minute readings in the presence of the proteinases are given in percentages of this value. The formol titration results are given in cc. of 0.02 *N* sodium hydroxide.

Table I shows the following facts. Trypsin inactivates the dehydrogenases at lower formol titration values than papain. The *d*-amino acid oxidase and the amine oxidase are inactivated more slowly than the other enzymes. The succinoxidase activity disappears more rapidly with trypsin than papain, although the

rate of inactivation of the cytochrome system is the same with the two proteinases. Experiments with methylene blue at corresponding times show that trypsin has not inactivated the dehydrogenase part of the succinoxidase system, whereas papain has caused some inactivation. These results indicate that some other protein is essential for the utilization of oxygen by the succinoxidase. Recent results by Stern and Melnick (2) and Hopkins, Lutwak-Mann, and Morgan (3) also indicate that this may be the case. Similar experiments suggest that this is also true for the choline oxidase.

TABLE I

*Effect of Incubation with Papain at pH 6.7 and Trypsin at pH 7.6 on Activity of Certain Dehydrogenases in Rat Liver and Kidney*

	Proteinase	Time of incubation	Activity	Formol titration, 0.02 N NaOH
		min.	per cent	cc.
Liver				
Succinoxidase	Papain	65	75	0.81
	Trypsin	65	29	0.65
Cytochrome oxidase	Papain	65	68	0.81
	Trypsin	65	59	0.65
Choline oxidase	Papain	65	70	0.81
	Trypsin	65	48	0.65
Amine oxidase	Papain	80	85	0.90
Kidney				
Succinoxidase	"	35	35	0.62
	Trypsin	35	6	0.38
Cytochrome oxidase	Papain	35	41	0.62
	Trypsin	35	40	0.38
d-Amino acid oxidase	Papain	40	86	0.70
	Trypsin	40	78	0.42

## DISCUSSION

Certain proteins present in the tissue preparations used are very sensitive to the action of papain and trypsin, even though neither enzyme was used at its optimum pH and the papain was used without cyanide. Inactivation of the succinoxidase in particular occurred in 60 to 90 minutes, when the formol titration values were only about 20 per cent of the values reached if the hydrolysis was allowed to proceed for 24 hours. This indicates



that certain essential proteins are very sensitive to hydrolytic activity and are inactivated when hydrolysis has proceeded only to a small degree.

## SUMMARY

1. The action of papain and trypsin on the succinoxidase, cytochrome-cytochrome oxidase system, choline oxidase, *d*-amino acid oxidase, and amine oxidase of rat liver and kidney has been studied.

2. The amine oxidase and the *d*-amino acid oxidase are inactivated much more slowly than the others by both proteinases.

3. The oxygen uptake but not the methylene blue reduction by the succinoxidase is inactivated more rapidly by trypsin than papain.

4. The cytochrome oxidase system is inactivated more slowly than the succinoxidase by the proteinases.

5. The succinoxidase and choline oxidase are inactivated at about the same rate.

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# THE DISTRIBUTION OF WATER AND ELECTROLYTES IN THE BLOOD OF DOLPHINS (*TURSIOPS* *TRUNCATUS*)\*

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The purpose of this paper is (1) to present data obtained from the analyses of whole blood and serum of dolphins; (2) to utilize these data to determine the distribution of water and electrolytes between the cells and the serum; and (3) to offer a comparison of these results with similar findings for dog and human blood.

Although great numbers of cetaceans (whales and dolphins) have been sacrificed for commercial purposes, little scientific information is available concerning the biology of this unique order. Particularly, the recorded analytical data for blood and tissues are scanty and for the most part unreliable, because of the difficulty of securing uncontaminated samples from living animals. Of the reports found in the literature, in only two instances was the blood taken from living animals (1, 2); the other reports were based on analyses of specimens obtained post mortem, mostly from the larger whales (3). There is no need to emphasize the difficulty of obtaining blood samples from large whales. The unavoidable lapse of time between the killing of these animals and the collection of the samples is sufficient to allow significant changes to take place in the blood; furthermore, there is great likelihood of con-

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† The authors are listed alphabetically because the work was carried out by them as a group.

tamination with sea water and body fluids. In view of the increasing interest in the biology of the cetaceans, it is important to have recorded analytical data obtained under controlled conditions.

### *Physiological Methods*

During the summer of 1939 we were enabled to secure live specimens of bottle-nose dolphins (*Tursiops truncatus*).<sup>1</sup>

From six bottle-nose dolphins six blood samples were taken under oil by heart puncture, with the animals out of water. Four of these samples were taken from unanesthetized animals and two were taken from animals anesthetized by the intraperitoneal injection of sodium phenobarbital.<sup>2</sup> One-half of the sample was immediately centrifuged under oil; the other half was defibrinated. The pH, carbon dioxide content, blood cell volume, and osmotic pressure were determined at the base laboratory, while the samples of serum and defibrinated blood were shipped to Chicago for complete analyses.

The blood samples were placed for shipment in unbreakable cellulose nitrate containers<sup>3</sup> of about 80 cc. capacity. The sample was cooled to approximately 5° in the plastic vial, which was covered with a rubber cap and sealed with adhesive tape. The vial was placed, tightly cushioned with rubber stoppers and cotton, in a precooled wide mouth thermos jar of pint capacity. The space between the vial and the jar was filled with crushed ice; the jar was then stoppered, packed in a corrugated carton, and shipped immediately by Air Express. Plastic containers were used in preference to glass to eliminate breakage in transit. The samples

<sup>1</sup> The collection of this material, as well as of a number of tissues, glands, and body fluids, was made possible through the whole-hearted cooperation of the scientific and technical staffs of the Marine Studios, Marineland, St. Augustine, Florida. The location of the Marine Studios is ideal for the capture of dolphins; the trained personnel handles these animals without injuring or unduly exciting them. The efficient service of the Eastern Air Lines and the Air Express of the Railway Express Agency was an indispensable link in the transportation of the samples from St. Augustine or Jacksonville to the laboratories in Chicago.

<sup>2</sup> All the animals so anesthetized died of respiratory failure within 1 hour after injection, even if only partially anesthetized with minimal doses of the drug.

<sup>3</sup> Obtained from Ivan Sorvall, 210 Fifth Avenue, New York.

reached the Chicago laboratories within 18 or 20 hours of the time of collection, cold and in excellent condition.

### *Chemical Methods*

All serum and blood analyses were made in duplicate. In order to obtain the most accurate results the composition of the red blood cells was determined from the analyses of the whole blood and the serum, and the accompanying measurement of cell volume. The following determinations were made on the serum: pH, total CO<sub>2</sub>, water, chloride, protein, albumin, globulin, sodium, potassium, calcium, magnesium, inorganic phosphorus, and osmotic pressure. On the whole defibrinated blood, determinations were made of cell volume, water, chloride, sodium, and potassium. In some of the experiments the whole blood was also analyzed for magnesium and oxygen capacity.

The pH of the serum was determined by an anaerobic glass electrode. Carbon dioxide content and oxygen capacity were measured with the Van Slyke and Neill manometric gas apparatus (4). Chlorides were determined by the wet ashing method of Van Slyke (5) with the Wilson and Ball modification (6); water, by weighing 2 cc. aliquots before and after drying to constant weight in platinum crucibles in a 100° oven; and inorganic phosphorus, by the Fiske and Subbarow method (7).

Proteins were determined by the micro-Kjeldahl method of Campbell and Hanna (8), the distillation of ammonia being carried out in the Goebel modification of the Pregl micro-Kjeldahl distillation apparatus (9). The proteins were estimated by multiplying by 6.25 the total nitrogen corrected for non-protein nitrogen (10). The albumin and globulin were determined by the method of Campbell and Hanna (11).

Sodium and potassium were determined by the methods of Butler and Tuthill (12) and Shohl and Bennett (13), respectively, with modifications which have already been described (14). Calcium determinations were made by the method of Kramer and Tisdall (15) as modified by Clark and Collip (16), and magnesium by the method of Denis (17) with modifications which have been reported in a previous paper (18). The amount of magnesium was estimated from the colorimetric determination of phosphate by the method of Fiske and Subbarow (7).

TABLE I  
Water and Electrolyte Concentrations in Dolphin Serum and Whole Blood

Dolphin No.	Sp. gr.	Osmotic pressure	Total CO <sub>2</sub>	pH	Cl	Na	K	Ca	Mg	Inorganic P	H <sub>2</sub> O	Cell vol-ume	Total protein	Non-protein N	Albumin to globulin ratio	O <sub>2</sub> capacity
	gm. per cc.	mM NaCl per l. H <sub>2</sub> O	mM per l.		mM per l.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.	gm. per l.	per cent	gm. per l.	gm. per l.		vol. per cent
Anesthetized	Serum	1.024	28.0	6.88	125.4	164.7	4.08	2.59	1.02	1.30	918.2		83.5	0.63	0.61	
	Blood	1.053			105.0	99.3	41.7				817.6	47.5				
	Serum	1.025	29.5	6.72	113.3	160.9	5.43	2.18	1.43	2.80	919.0		85.6	0.49	0.60	
	Blood	1.055			94.7	93.6	47.9				823.0	45.8				19.1
Unanesthetized	Serum	1.023	29.8	7.30	115.3	155.4	4.03	2.49	1.23	2.24	930.3		73.4	0.48	0.74	
	Blood	1.057			92.3	92.8	44.8				828.3	44.1				
	Serum	1.021	31.9	7.46	105.1	149.1	3.52	2.00	1.06	1.08	926.3		76.1	0.35	0.72	
	Blood	1.047			80.7	91.8	42.7				822.1	43.0				
	Serum	1.024			107.5	154.0	5.00	2.40	1.06		924.7		79.1	0.57	0.87	
	Blood				87.5	97.1	45.3		2.27		834.4	41.2				19.6
	Serum	1.025			112.3	154.6	4.71	2.35	1.09		928.5		79.9	0.53	1.31	
	Blood	1.052			91.3	97.0	43.4		2.94		838.8	39.0				19.0
	Serum	1.023			110.2	153.3	4.32	2.31	1.11		927.4		77.6	0.52	0.90	
	Blood	0.001			4.0	2.4	0.59	0.18	0.14		2.2		2.6	0.10	0.23	
	Mean	1.051			88.0	94.7	44.1				830.9	41.8				
	$\sigma^*$	0.001			4.5	2.4	1.04				6.3	1.9				
10 normal dogs (Eichelberger, unpublished data)																
Mean	Serum		25.2	7.40	108.5	145.4	4.00	2.50			937.9		61.9	0.34	2.17	
$\sigma^*$			2.4	0.06	4.1	4.1	0.62	0.02			3.1		4.6	0.02	0.5	
Mean	Blood				87.0	121.2	6.48				827.3	46.8				
$\sigma^*$					5.5	6.5	0.60				10.8	5.4				

\* Standard deviation.

TABLE II

*Concentration of Water and Electrolytes in Serum and Cells*

Calculated from the data of Table I. The units are expressed per liter.

Dolphin No.		H <sub>2</sub> O	Cl	Na	K	Mg
Anesthetized dolphins						
		<i>gm.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>
2	Serum	918.2	125.4	164.7	4.08	
	Cells	705	82.6	26.9	83.4	
4	Serum	919.0	113.3	160.9	5.43	
	Cells	707	72.6	14.2	98.2	
Unanesthetized dolphins						
1	Serum	930.3	115.3	155.4	4.03	
	Cells	700	63.5	13.4	96.5	
3	Serum	926.3	105.1	149.1	3.52	
	Cells	685	48.2	15.8	94.8	
5	Serum	924.7	107.5	154.0	5.00	2.12
	Cells	705	59.0	15.8	102.8	8.02
6	Serum	928.5	112.3	154.6	4.71	2.18
	Cells	700	59.3	7.7	103.5	11.65
Mean*.....	Serum	927.4	110.2	153.3	4.32	2.15
$\sigma$ .....		2.2	4.0	2.4	0.59	0.03
Mean.....	Cells	698	57.5	13.2	99.4	9.83
$\sigma$ .....		7.5	5.6	3.3	3.8	1.8
10 normal dogs (Eichelberger, unpublished data)						
Mean.....	Serum	937.9	108.5	145.4	4.00	
$\sigma$ .....		3.1	4.1	4.1	0.62	
Mean.....	Cells	827.2	62.3	93.9	9.37	
$\sigma$ .....		0.8	6.4	7.7	1.1	
10 humans (Hald and Eisenman (20))						
Mean.....	Serum	937.3	102.5†	135.5	4.29	
$\sigma$ .....		1.8	1.0	2.6	0.50	
Mean.....	Cells	404.8		17.7	81.8	
$\sigma$ .....		3.6		4.3	5.0	

\* Mean for the four unanesthetized animals only.

† Chloride from data of Hastings *et al.* (21).

Osmotic pressure was determined with a modified Baldes thermocouple (19). The difference was found between the vapor pressures of the sample and of a solution of a known concentration

of sodium chloride, measured at  $37.5^{\circ}$  in an atmosphere of 95:5,  $O_2:CO_2$ ; and the osmotic pressures were expressed in terms of the molality of sodium chloride equivalent to the vapor pressure of the sample, corrected for oxygen and carbon dioxide solubilities.

### Results

In Table I are given experimental results from two groups of animals: (1) those anesthetized with sodium phenobarbital (Dolphins 2 and 4) and (2) those which were unanesthetized (Dolphins 1, 3, 5, and 6).

It will be observed that all values for both serum and whole blood of the unanesthetized dolphins were notably constant, and that the variations of the water content and the concentrations of inorganic constituents of the whole blood were not significantly different from the variations of the same constituents of serum.

From the analytical data given in Table I the distribution of water and electrolytes in the red cells has been calculated; the calculations are presented in Table II. It will be noted that while the range of variation of water and electrolyte concentrations in the serum of the unanesthetized animals was relatively small, this was not true for the cells. The total base of the red cell was composed principally of potassium and sodium, the potassium value being 99.4 mm,  $\pm 3.8$ , and the sodium value being 13.2 mm,  $\pm 3.3$ . The high value for sodium in the serum (153.3 mm,  $\pm 2.4$ ) is decisively different from that found for land mammals.

### DISCUSSION

The analytical data presented in this paper were obtained with blood from live animals under conditions which precluded contamination or postmortem changes. Previously recorded data secured in the study of dead whales are not comparable to our results. The only figures comparable with ours are those reported by Green and Redfield (2) for the oxygen capacity of blood and the carbon dioxide content of serum from one living porpoise (*Phocaena phocaena*). Their value for oxygen capacity (22.18 volumes per cent) is similar to ours (19.1 volumes per cent); and their value for carbon dioxide content (45 volumes per cent, or 20.21 mm) is lower than the value of 30.9 mm per liter that we obtained for unanesthetized dolphins.

Except for the high values for sodium and the low albumin to globulin ratios, the values for the inorganic cations and all other constituents in the dolphin serum agree within experimental limits with those for dog serum (Table I). This similarity, however, does not hold for the red cells. The total base of the red cells of the dolphin is composed mainly of potassium, while in the red cells of the dog (Table II) it is chiefly sodium; the red cells of the dolphin thus more nearly resemble those of man. It is to be noted that the potassium concentration in the dolphin red cells is even higher than that in human red cells (Table II). The serum osmotic pressures were higher for the dolphin than for man, for Margaria gives a value for the latter of 158.6 mm of sodium chloride per liter of water (22).

These findings suggest that the blood system of the bottle-nosed dolphin is not appreciably different from that of terrestrial mammals. In this respect the adaptation which these animals have undergone is less pronounced than the structural changes which the cetaceans underwent in order to make their existence compatible with an aquatic environment (23). Further experimentation must be carried out before it can be decided whether this statement is tenable for other cetaceans and aquatic mammals.

#### SUMMARY

The concentrations of water and electrolytes in the serum and red cells of four living, unanesthetized dolphins have been estimated from the analyses of serum and whole blood and from measurements of cell volume.

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## THE ACTIVATION OF PAPAIN

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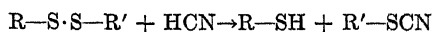
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In previous reports (1, 2) there was presented evidence which could not be fitted into the widely accepted oxidation-reduction theory of papain activation (3). In these experiments it was shown that papain may be inhibited by means of phenylhydrazine and may be reactivated by means of benzaldehyde. In an attempt to study further the process of papain activation, an inactive papain preparation (Papain A) was activated by HCN (HCN-Papain A) and subsequently precipitated by means of isopropyl alcohol. The activity of the enzyme preparation was determined before and after this precipitation. If the activation of papain by HCN consisted simply in the reduction of disulfide groups of the enzyme to sulfhydryl groups, then the precipitated Papain B without added activator should have exhibited approximately the same activity as did the HCN-papain before precipitation. However, the precipitate was found to behave like an unactivated papain, since it was completely inactive toward carbobenzoxy-isoglutamine and only slightly active toward benzoylarginineamide. The oxidation-reduction theory of papain activation offers no simple explanation for this result.

Furthermore, the precipitate, Papain B, on addition of HCN, regained nearly all of the activity of the original HCN-Papain A. This activated Papain B was precipitated a second time by means of isopropyl alcohol, resulting in an inactive enzyme preparation, Papain C, which, on addition of HCN, regained the activity of the original HCN-Papain A. It is difficult to interpret the nearly complete recovery of the original activity after two inactivations and reactivations by HCN in terms of the disulfide-sulfhydryl

theory. It is well known (4) that the action of HCN on disulfide linkages yields only one sulfhydryl group.



The action of the various papain preparations discussed above upon two synthetic substrates is summarized in Table I. The preparations were tested without added activator, with HCN, and also with cysteine as activator.

TABLE I  
*Hydrolysis of Synthetic Substrates by Papain Following Isopropyl Alcohol Precipitation*

Papain solution	Enzyme solution per 2.5 cc.	Added activator	Hydrolysis			
			Carbobenzoxyisoglutamine		Benzoylarginineamide	
			2 hrs.	4 hrs.	2 hrs.	4 hrs.
	cc.		per cent	per cent	per cent	per cent
A	0.3	None	1	0	6	7
B	0.3		1	1	9	13
C	0.3		-1	0	4	9
A	0.3	HCN	35	54		
B	0.3		29	49		
C	0.3		27	48		
A	0.15	Cysteine	22	29	46	61
B	0.15		22	28	40	55
C	0.15		19	27	36	52
A	0.3		42	58		
B	0.3		43	57		
C	0.3		41	58		
A	0.15		22	28	65	79
B	0.15		25	30	68	86
C	0.15		22	29	66	82

These experiments may perhaps be best explained by means of the hypothesis that the HCN combines with the "inactive" papain to form a dissociable HCN-papain compound which represents the HCN-activated enzyme.<sup>1</sup> On precipitation of the enzyme by means of isopropyl alcohol, the HCN-enzyme compound dissociates and the precipitate contains the HCN-free

<sup>1</sup> Maschmann (5) has observed that the inactivation of papain by means of iodoacetic acid is reversed upon precipitation of the enzyme by alcohol. This finding may also be interpreted as the dissociation of an inactive papain-inhibitor compound.

enzyme that is inactive towards synthetic substrates. Correspondingly, it may be assumed that the activation by cysteine consists in the formation of a dissociable cysteine-papain compound, and the activation with glutathione in the formation of a glutathione-papain compound. More generally, each of the various activators combines with the same inactive enzyme to form a different activator-enzyme compound. On the basis of this theory it may be expected that the specificity of the various activator-enzyme compounds should differ, depending upon the nature of the activator applied. This expectation seems to be fulfilled in the experiments with benzoylarginineamide reported in Table I. The activation with cysteine produces an essentially greater enzymatic activity than does the activation with HCN. Dr. George W. Irving, Jr., of this laboratory has made similar observations regarding the action of HCN-papain and cysteine-papain towards carbobenzoxy-leucylglycylglycine. In this case, HCN-papain possesses the greater activity. In these activations papain appears to act as an apoenzyme which is activated by coenzymes such as HCN, cysteine, or glutathione.

Mendel and Blood have already found that the activation of papain by HCN with egg white as substrate could be reversed by aeration or dialysis. After excluding other possibilities, these authors concluded that, pending further investigation, nothing remained but to compare the behavior of HCN with that of the coenzymes (6). Although this point of view received strong support from Willstätter and Grassmann (7), it was pushed into obscurity with the acceptance of the oxidation-reduction theory. It now seems necessary to return to the coenzyme theory of Mendel and Blood, and to investigate its validity for the activation of intracellular proteolytic enzymes other than papain.

The availability of simple synthetic substrates makes possible a detailed study of the specificities of the various activator-enzyme compounds. The fact that the various activators may produce different specificities should play a significant rôle in the biological action of the intracellular enzymes.

#### EXPERIMENTAL

*Papain A*—An aqueous solution of 225 mg. of purified papain (8) in 25 cc. (to be referred to as the solution of Papain A) was tested for its enzymatic activity by diluting 1.25 cc. of this solution to

2.5 cc. through the addition of 0.5 cc. of water and 0.75 cc. of 0.2 M citrate buffer (pH 5). Similarly, the effect of HCN was determined by diluting 1.25 cc. of the papain solution to 2.5 cc. with 0.5 cc. of 0.46 M HCN solution (pH 5) and 0.75 cc. of citrate buffer. The HCN-papain mixture was incubated for 2 hours at 40° before being tested. Cysteine-papain was prepared by diluting 1.25 cc. of the papain solution to 2.5 cc. with 0.5 cc. of 0.2 M cysteine solution (pH 5) and 0.75 cc. of citrate buffer. 0.3 or 0.15 cc. of these enzyme solutions was employed per 2.5 cc. of test solution, as indicated in Table I. The substrate concentration was 0.05 mM per cc. of test solution. The extent of hydrolysis was followed by measuring the liberated carboxyl groups according to the method of Grassmann and Heyde (9). The temperature in all cases was 40°, and the pH of the test solution was maintained at 5.0.

*Papain B*—20 cc. of the above solution of Papain A were mixed with 8 cc. of 0.46 M HCN solution and incubated at 40° for 2 hours. The mixture was then chilled and 100 cc. of cold isopropyl alcohol were added. After it had been allowed to stand at 5° for 30 minutes, the precipitate was centrifuged down and washed once with 50 cc. of cold isopropyl alcohol. The centrifuged precipitate was dissolved in water to a volume of 20 cc. This will be referred to as the solution of Papain B. Three 1.25 cc. aliquots were removed from this enzyme solution and tested without added activator, with HCN, and with cysteine, as described for Papain A.

*Papain C*—15 cc. of the solution of Papain B were incubated with 6 cc. of 0.46 M HCN solution for 2 hours and, after chilling, mixed with 100 cc. of cold isopropyl alcohol. The resulting precipitate was centrifuged and washed as in the case of Papain B, and its enzymatic activity tested as described for Papain A.

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## THE OCCURRENCE OF LEUCYLPEPTIDASE\*

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In hog erepsin, the presence of at least two distinct polypeptidases, namely leucylpeptidase and aminopolypeptidase, appears to be certain. These have been prepared in enzymatically pure condition and their properties studied (1-3). In the case of leucylpeptidase, leucylglycine and leucyldiglycine are hydrolyzed rapidly and at equal rates, but alanyl and glycyl peptides are split very slowly. The hydrolysis of all the substrates split by leucylpeptidase is activated by Mg and Mn ions. No metal activator has been found for hog aminopolypeptidase, but it may be characterized by its unusually rapid hydrolysis of prolyldiglycine and triglycine, which are split only very slowly by leucylpeptidase.

Magnesium activation of leucylglycine hydrolysis by peptidases of *Tubifex* eggs has been reported by Holter *et al.* (4), but no data were given for leucyldiglycine hydrolysis. Magnesium activation of the hydrolysis of leucyldiglycine but not leucylglycine by peptidases of *Clostridium histolyticum* was reported by Kocholaty *et al.* (5). A hog leucylpeptidase-like enzyme has been shown to exist in three plants (6) and in some bacteria (7). Abderhalden and Hanson (8) have presented evidence for the presence of a leucylpeptidase in bovine eye lens and cornea. In both cases the hydrolysis of leucylglycine was activated by magnesium ions and, with the lens extracts, leucylglycine and leucyldiglycine were split at equal rates.

In the present paper, leucylpeptidases are shown to be present in

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the proteolytic enzyme systems of human duodenum, rat intestine, rat carcinoma tissue, chick intestine, trout tissues, lobster tissues, cockroach intestine, and the bacteria *Pseudomonas fluorescens* and *Rhizobium trifolii*, but not in brewers' yeast or molds.

#### EXPERIMENTAL

##### *Methods*

Crude enzyme extracts were generally made as follows: Fresh tissues were ground with sand, then suspended in 4 to 5 times their weight of 20 per cent by volume aqueous glycerol, and allowed to extract, without pH adjustment, in the presence of toluene for 4 to 24 hours. The extracts were clarified before use by centrifugation or filtration with Hyflo super-cel.<sup>1</sup> Enzyme extracts were made from the bacteria, molds, and yeast as previously described (3, 7). Cells of *Rhizobium trifolii*, Strain 209, were grown on twenty-eight 1 liter Roux bottle agar plates containing 100 cc. of medium of the following composition: MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 gm., NaCl 0.1 gm., CaCO<sub>3</sub> 0.5 gm., K<sub>2</sub>HPO<sub>4</sub> 0.5 gm., agar 25.0 gm., yeast water<sup>2</sup> 250 cc., distilled water 750 cc. The pH was adjusted to 6.8 to 7.0.

After 64 hours growth at 28°, the cells were washed off the surface of the agar and collected by centrifuging. The yield was 4.5 gm. of fresh cells.<sup>3</sup> These were frozen and thawed repeatedly over a period of 2½ months. They were then suspended in 30 cc. of water at pH 7 in the presence of toluene and allowed to autolyze for 6 days. Daily analyses for leucyldiglycine-hydrolyzing enzymes indicated maximum enzyme liberation at this time. The suspension was clarified by centrifuging and the clear extract analyzed.

Details concerning the substrates and methods used to determine the activity of the enzyme preparations have been given in a previous paper (3).

##### *Results*

The similarity of the intestinal peptidase systems of various mammals has generally been assumed, but lack of suitable identi-

<sup>1</sup> A filter aid sold by Johns-Manville.

<sup>2</sup> The clear extract from 200 gm. of pressed yeast autoclaved in 1 liter of water.

<sup>3</sup> We wish to thank Dr. R. H. Burris for growing these cells.

fying characteristics has hitherto made it difficult to prove this assumption. Evidence for such similarity is given in the data of this paper.

Table I presents the data for the activities of crude and purified peptidase solutions obtained from human duodenum. Crude extracts hydrolyzed all six peptides rapidly. A freshly precipitated acetone preparation contained a leucylpeptidase (indicated by Mg activation of leucylglycine and leucyldiglycine hydrolysis) contaminated with a considerable amount of dipeptidase activity.

TABLE I  
*Peptidases of Human Duodenum*

Substrate	Crude extract*		Acetone-precipitated enzyme†				Relative activities (leucylglycine = 1.0)		
	Time of incubation	Hydrolysis	Enzyme per 3 cc. reaction mixture	Time of incubation	Hydrolysis		Human leucylpeptidase		Hog leucylpeptidase
					No activator	0.003 M MgCl <sub>2</sub>	Fresh	Aged	
	min.	per cent	cc.	min.	per cent	per cent			
<i>dl</i> -Alanylglycine....	14.5	78	0.05	60	64	68	3.6	0.3	0.1
<i>dl</i> -Leucylglycine....	60	60	0.2	60	64	76	1.0	1.0	1.0
Diglycine.....	40	52	0.5	39	40	42	0.3	0.03	0.003
<i>dl</i> -Alanyldiglycine..	30	60	1.0	120	24	46	0.06	0.2	0.2
<i>dl</i> -Leucyldiglycine..	120	48	1.0	35	41	70	0.3	1.0	1.0
Triglycine.....	120	58	1.0	120	8	10	0.01	0.03	0.003

\* 0.1 cc. of enzyme solution (equivalent to 0.02 mg. of fresh tissue) was used in 3 cc. of reaction mixture for every peptide except diglycine, for which 0.5 cc. was used.

† 60 cc. of crude extract at pH 6.1 were precipitated with 60 cc. of acetone and the portion of the precipitate soluble in 60 cc. of H<sub>2</sub>O was analyzed.

However, when the preparation was allowed to age for 21 days at 2°, 98 per cent of the alanylglycine- and diglycine-splitting activity and 77 per cent of the leucylglycine-splitting activity were lost. The dipeptide-splitting activity that remained was evidently due to the leucylpeptidase, since in the presence of 0.003 M MgCl<sub>2</sub>, alanylglycine and leucylglycine were hydrolyzed to the extent of 76 and 86 per cent respectively, compared to 22 and 48 per cent hydrolysis in the absence of Mg ions. Leucylglycine and leucyldiglycine were hydrolyzed at equal rates by this aged



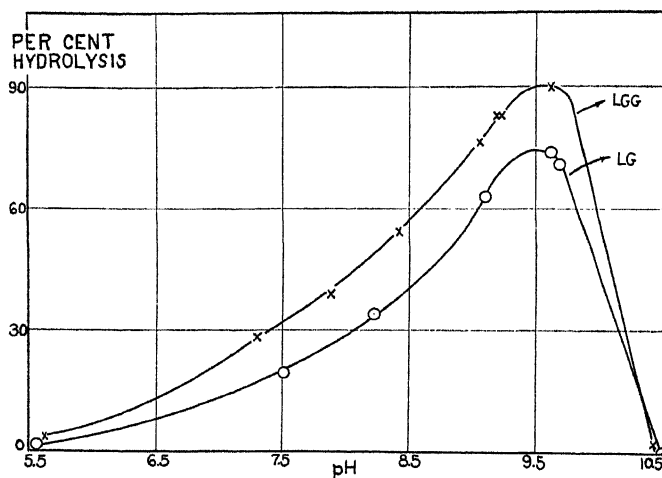


FIG. 1. pH dependence of human intestinal leucylpeptidase activity. 0.5 cc. of an acetone-precipitated enzyme preparation was used in 3 cc. of reaction mixture for leucylglycine analyses. The incubation time was 1 hour. 0.003 M  $\text{MgCl}_2$  was present in all determinations. LG represents leucylglycine; LGG, leucyldiglycine.

TABLE II  
Peptidases of Rat Intestine

Substrate	Crude extract*			Dialyzed acetone-precipitated enzyme†					
	En- zyme per 3 cc. reaction mixture	Time of incu- pation	Hy- dro- lysis	En- zyme per 3 cc. reaction mixture	Time of incu- pation	Hydrolysis			Rela- tive ac- tivities (leucyl- glycine = 1.0)
						No acti- vator	0.01 M $\text{MgSO}_4$	0.001 M $\text{MnSO}_4$	
	cc.	min.	per cent	cc.	hrs.	per cent	per cent	per cent	
<i>dl</i> -Alanylglycine....	0.25	50	52	0.1	1	19	28	46	0.5
<i>dl</i> -Leucylglycine....	0.10	50	56	0.025	3	12	51	66	1.0
Diglycine.....	0.20	60	22	0.3	2	46	48	52	0.1
<i>dl</i> -Alanyldiglycine..	0.05	30	60	0.1	2	47	64	76	0.4
<i>dl</i> -Leucyldiglycine..	0.10	50	60	0.025	3	12	38	70	1.1
Triglycine.....	0.10	50	56	0.3	1	36	38	28	0.1

\* 1 cc. of crude extract was equivalent to 0.18 gm. of fresh tissue. Determinations were made in the presence of 0.003 M  $\text{MgCl}_2$ .

† 60 cc. of crude extract (a different preparation), at pH 6.7, were diluted to 120 cc. with  $\text{H}_2\text{O}$  and mixed with 120 cc. of acetone. The resulting precipitate was centrifuged down and suspended in 15 cc. of  $\text{H}_2\text{O}$ . The suspension was then dialyzed against distilled  $\text{H}_2\text{O}$  for 12 hours at  $2^\circ$ , and clarified by centrifuging before analysis.

preparation and the activity ratios in general were very similar to those of hog leucylpeptidase.

Fig. 1 shows graphically the pH optima for the hydrolysis of leucylglycine and leucyldiglycine by a fresh, acetone-precipitated preparation from human duodenum. Both peptides were hydrolyzed most rapidly at pH 9.4 to 9.6. In the case of leucylglycine, the hydrolysis was probably due to a mixture of leucylpeptidase and a dipeptidase, as discussed above.

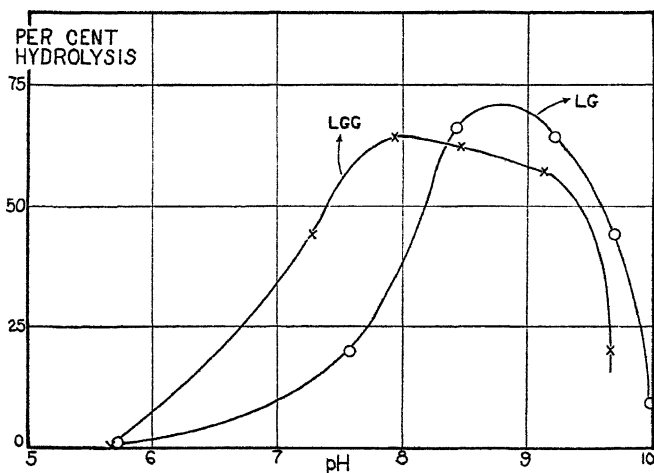


FIG. 2. pH dependence of rat intestinal leucylpeptidase activity. 0.6 cc. of an acetone-precipitated enzyme preparation was used in 3 cc. of reaction mixture for leucyldiglycine (*LGG*) analyses and 0.3 cc. for leucylglycine (*LG*) analyses. The incubation time was 1 hour. 0.003 M  $MgCl_2$  was present in all determinations.

The second mammalian intestinal system studied was that of the rat. The data are given in Table II. Crude extracts hydrolyzed all six peptides rapidly, thus resembling hog and human crepsins. The presence of a leucylpeptidase was evident from the typical Mg and Mn ion activation of leucylglycine and leucyldiglycine hydrolysis. The relative activities of the acetone-precipitated preparation approximated those of hog leucylpeptidase. Crude rat erepsin hydrolyzed prolyldiglycine (0.1 M, pH 8) 1.2 times as fast as triglycine. Since this property has been shown to be characteristic of purified hog aminopolypepti-

dase (2), it strongly suggests the presence of such an enzyme in rat erepsin.

The pH-activity curves for the hydrolysis of leucyldiglycine and leucylglycine by rat leucylpeptidase are shown in Fig. 2. The curve for leucyldiglycine splitting is very similar to the one given by hog leucylpeptidase (1). Leucylglycine was hydrolyzed most rapidly at pH 8.8.

TABLE III  
*Peptidases of Rat Carcinoma Tissue*

Substrate	Crude extract*			Acetone-precipitated enzyme†				
	En- zyme per 3 cc. reaction mixture	Hydrolysis		Time of in- cuba- tion	Hydrolysis			Rela- tive ac- tivities (leucyl- glycine = 1.0)
		No acti- vator	0.001 M MnSO <sub>4</sub>		No acti- vator	0.01 M MgSO <sub>4</sub>	0.001 M MnSO <sub>4</sub>	
	cc.	per cent	per cent	hrs.	per cent	per cent	per cent	
<i>dl</i> -Alanylglycine.....	0.2	15	15	2	29	54	50	0.05
<i>dl</i> -Leucylglycine....	0.1	18	39	1	8	19	32	1.0
Diglycine.....	0.5	7	8	4	3	8	4	0.004
<i>dl</i> -Alanyldiglycine..	0.05	48	30	2	28	71	52	0.07
<i>dl</i> -Leucyldiglycine..	0.1	21	42	1	10	21	34	1.0
Triglycine.....	0.1	42	29	4	3	11	4	0.005

\* 1 cc. of extract was equivalent to 0.21 gm. of fresh tissue. The incubation time was 1 hour.

† 150 cc. of crude extract were adjusted to pH 6.02, 150 cc. of acetone added, and the mixture allowed to stand at room temperature for 1.5 hours. The resulting precipitate was centrifuged down, suspended in 50 cc. of H<sub>2</sub>O at pH 6.85, and the suspension centrifuged. 1.0 cc. of clear extract was used in 3 cc. of reaction mixture, except for leucylglycine and leucyldiglycine analyses for which 0.06 cc. was used.

The peptidase system of a Flexner-Jobling rat carcinoma<sup>4</sup> was next investigated for the presence of leucylpeptidase. As shown by the data of Table III, rat carcinoma tissue contains a leucylpeptidase almost identical in specificity with the purest hog leucylpeptidase.

The presence of a hog aminopolypeptidase-like enzyme in the crude extracts was indicated by the fact that prolyldiglycine was split 1.2 times as fast as triglycine. Crude extracts were unable

<sup>4</sup> We wish to thank Dr. C. A. Baumann for supplying the tumor tissue.

to hydrolyze appreciably either *d*-leucylglycine or *d*-leucyldiglycine (the racemic mixtures were split at least 150 times as rapidly). Peptidases activated by reducing agents, such as have been found in anaerobic bacteria and elsewhere (3, 9), appeared to be absent, as indicated by the non-activation of alanyldiglycine and leucyldiglycine hydrolysis by either 0.001 M concentration of the reducing agent *p*-methylaminophenol sulfate or by 0.001 M ZnSO<sub>4</sub>.

TABLE IV  
*Peptidases of Chick Intestine*

Substrate	Crude extract*		Acetone-precipitated enzyme†				
	Enzyme per 3 cc. reaction mixture	Hy- drolysis	Time of in- cuba- tion	Hydrolysis			Relative activities (leucyl- glycine = 1.0)
				No acti- vator	0.01 M MgSO <sub>4</sub>	0.001 M MnSO <sub>4</sub>	
	cc.	per cent	hrs.	per cent	per cent	per cent	
<i>dl</i> -Alanylglycine.....	0.005	48	4	19	35	48	0.2
<i>dl</i> -Leucylglycine.....	0.05	86	1	8	17	50	1.0
Diglycine.....	0.15	76	4	6	10	11	0.05
<i>dl</i> -Alanyldiglycine.....	0.005	20	6	35	60	56	0.2
<i>dl</i> -Leucyldiglycine.....	0.05	40	2	14	48	68	0.7
Triglycine.....	0.05	76	6	16	16	22	0.07

\* 1 cc. of crude extract was equivalent to 0.21 gm. of wet tissue. The substrates all contained 0.003 M MgCl<sub>2</sub>. The incubation time was 1 hour.

† 50 cc. of crude extract (a different preparation) were mixed at pH 6.45 with 50 cc. of acetone and allowed to stand at room temperature for 1.5 hours. The resulting precipitate was centrifuged down, suspended in 50 cc. of water, and the suspension clarified by centrifuging. After aging for 10 days at 2°, the solution was analyzed. 1.0 cc. was used in 3 cc. of reaction mixture in all cases.

In Table IV the data obtained with the peptidase system of chick intestine demonstrate the presence of an Mg-Mn-activated leucylpeptidase. In order to remove considerable amounts of dipeptidase and non-activatable polypeptidase activities, a long treatment with acetone had to be used. This yielded a preparation with specificity and metal activatability properties quite similar to those of hog leucylpeptidase. In the presence of 0.005 M MgSO<sub>4</sub>, the acetone-precipitated preparation hydrolyzed 0.05 M leucyldiglycine 1.2 times as rapidly as 0.05 M glycyl-*dl*-leucyl-

glycine. Chick leucylpeptidase hydrolyzes leucyldiglycine most rapidly at pH 8.7, as shown in Fig. 3.

The usual acetone precipitation technique did not yield very pure leucylpeptidase preparations from either the intestine or liver of the trout (*Salmo irideus*), as shown in Tables V and VI. In both cases, alanylglycine- and diglycine-splitting enzymes appeared to withstand the acetone treatment very well. How-

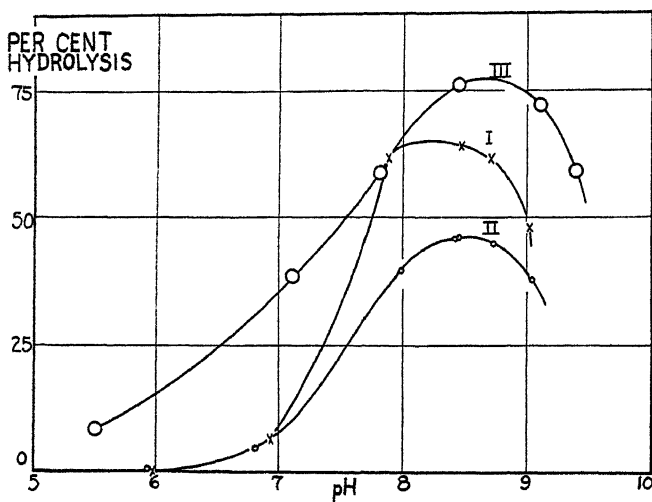


FIG. 3. pH dependence of leucyldiglycine hydrolysis by trout and chick leucylpeptidases. 0.08 cc. of an acetone-precipitated preparation from trout intestine (Curve I) and 0.10 cc. of an acetone-precipitated preparation from trout liver (Curve II) were used in 3 cc. of leucyldiglycine reaction mixture which contained 0.001 M  $\text{MnSO}_4$ . For the determinations with chick leucylpeptidase (Curve III), 0.88 cc. of an acetone-precipitated preparation was used in 3 cc. of a reaction mixture containing 0.003 M  $\text{MgCl}_2$ .

ever, the presence of a leucylpeptidase in both tissues appears certain, since the leucyl peptides were split at equal rates (in the presence of Mn ions) and their rates of hydrolysis were accelerated by Mg and Mn ions. It may be seen that the hydrolysis of alanylglycine and diglycine by the crude intestinal extract but not by the acetone-precipitated preparations was activated by  $\text{MgSO}_4$ . However, several dipeptidases may be present, since it

has been possible to obtain acetone-precipitated preparations which split diglycine, but not alanylglycine, more rapidly in the presence of  $MgCl_2$ .

Crude extracts of trout liver and intestine hydrolyzed prolyldiglycine about 2.5 times as fast as triglycine, thus differing substantially from hog aminopolypeptidase. This difference might

TABLE V  
*Peptidases of Trout Intestine*

Substrate	Crude extract*					Hydrolysis by acetone-precipitated enzyme†		
	Enzyme per 3 cc. reaction mixture	Hydrolysis				No activator	0.001 M $MnSO_4$	0.01 M $MgSO_4$
		No activator	0.001 M $MnSO_4$	0.01 M $MgSO_4$	0.001 M $CoCl_2$			
	cc.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
<i>dl</i> -Alanylglycine . . .	0.25	28	72	54	29	33	78	34
<i>dl</i> -Leucylglycine . . .	0.08	28	63	37	27	30	55	36
Diglycine . . . . .	0.25	18	36‡	48	40	7	50	7
<i>dl</i> -Alanyldiglycine . .	0.025	28	28			10	30	14
<i>dl</i> -Leucyldiglycine . .	0.20	37	44§	51	34	8	54	12
Triglycine . . . . .	0.12	30	45			5	58	5

\* 1.0 cc. of extract was equivalent to 0.20 gm. of fresh tissue. The incubation time was 1 hour, except for diglycine (2 hours).

† 20 cc. of crude extract at pH 6.2 were mixed with 20 cc. of acetone and the mixture centrifuged after 3 minutes; the resulting precipitate dissolved completely in 20 cc. of  $H_2O$  at pH 7.15. Of this solution, 0.6 cc. was used in 3 cc. of reaction mixture for alanylglycine, alanyldiglycine, and triglycine analyses, and 0.3 cc. was used for the three remaining peptides. The incubation time was 0.5 hour, except for triglycine (1 hour). In the presence of 0.001 M  $CoCl_2$ , 30 per cent splitting of alanylglycine and 12 per cent splitting of diglycine were obtained.

‡ 0.025 cc. of enzyme solution.

§ 0.05 cc. of enzyme solution.

be caused by accompanying substances, or by the presence of an enzyme other than leucylpeptidase or aminopolypeptidase, which hydrolyzes prolyldiglycine very rapidly.

Leucylpeptidase-like enzymes, as indicated by Mg activation of leucylglycine and leucyldiglycine hydrolysis, have also been found in the spleen, kidney, and ceca of the trout. The relative abund-

ance of leucylglycine-hydrolyzing enzymes in trout tissues was as follows: spleen 400, liver 190, kidney 148, intestine 93, and ceca 92.

From Fig. 3 it may be seen that leucylpeptidase preparations (acetone-precipitated extracts) from trout intestine and trout liver hydrolyzed leucyldiglycine most rapidly at pH 7.9 to 8.8 and pH 8.2 to 8.8 respectively.

TABLE VI  
*Peptidases of Trout Liver*

Substrate	Hydrolysis by crude extract*	Acetone-precipitated enzyme†				
		Enzyme per 3 cc. reaction mixture	Time of incubation	Hydrolysis		
				No activator	0.001 M MnSO <sub>4</sub>	0.01 M MgSO <sub>4</sub>
	per cent	cc.	hrs.	per cent	per cent	per cent
<i>dl</i> -Alanylglycine.....	62	0.1	1.0	22‡	66	29
<i>dl</i> -Leucylglycine.....	34	0.1	1.5	44	68	52
Diglycine.....	23	0.5	0.5	23‡	60	24
<i>dl</i> -Alanyldiglycine...	34	0.5	1.5	5	43	10
<i>dl</i> -Leucyldiglycine...	66	0.2	1.0	24	62	30
Triglycine.....	19	0.5	1.5	5	34	10

\* 0.25 cc. of crude extract (equivalent to 0.05 gm. of wet tissue) was used in 3 cc. of reaction mixture, except for leucylglycine (0.025 cc.). The time of incubation was 0.5 hour, except for leucylglycine and triglycine (1 hour).

† 33 cc. of crude extract at pH 6.1 were mixed with 33 cc. of acetone and the mixture allowed to stand at room temperature for 1 hour. The resulting precipitate was centrifuged down, suspended in 33 cc. of H<sub>2</sub>O at pH 6.8, and the suspension centrifuged. The clear solution was analyzed.

‡ 0.001 M CoCl<sub>2</sub> did not activate the hydrolysis of either alanylglycine or diglycine.

The bacteria *Pseudomonas fluorescens* and *Proteus vulgaris* have previously been reported to contain leucylpeptidases whose leucylglycine and leucyldiglycine hydrolyses occurred at equal rates and which were activated by Mg ions (7). The specificity of the *Proteus* leucylpeptidase (later shown to be activated by Mn as well as Mg ions (3)) was similar to that of hog leucylpeptidase, but the *Pseudomonas* preparation hydrolyzed alanyl and glycyl peptides almost as rapidly as leucyl peptides. It was

desirable to determine whether *Pseudomonas* leucylpeptidase could hydrolyze glycyl peptides rapidly or whether some contaminating enzymes had been present in the former preparations. The data obtained with a new batch of cells grown and extracted as previously described (7) are summarized in Table VII. It may be seen that with the proper acetone treatment the glycyl and alanyl peptide-splitting enzymes are destroyed and the re-

TABLE VII  
*Peptidases of Pseudomonas fluorescens*

Substrate	Crude enzyme*		Acetone-precipitated enzyme†				
	Time of incubation	Hydrolysis	Time of incubation	Hydrolysis			Relative activities‡ (leucylglycine = 1.0)
				No activator	0.01 M MgSO <sub>4</sub>	0.001 M MnSO <sub>4</sub>	
	hrs.	per cent	hrs.	per cent	per cent	per cent	
<i>dl</i> -Alanylglycine.....	1	12	3	10	20	40	0.2
<i>dl</i> -Leucylglycine.....	1	30	1	8	32	36	1.0
Diglycine.....	18	28	3	3	6	6	0.03
<i>dl</i> -Alanyldiglycine.....	1	10	3	8	18	38	0.2
<i>dl</i> -Leucyldiglycine.....	1	36	1	10	36	62	1.7
Triglycine.....	18	34	3	4	8	8	0.03

\* 0.5 cc. of crude enzyme solution (equivalent to 0.065 gm. of wet cells) was used in 3 cc. of reaction mixture, except for leucylglycine and leucyldiglycine (0.25 cc.). The substrates contained 0.001 M MnSO<sub>4</sub>.

† 30 cc. of crude extract at pH 6.3 were precipitated with 30 cc. of acetone; the precipitate was suspended in 11 cc. of H<sub>2</sub>O at pH 7.0, and the suspension clarified by centrifuging. 0.5 cc. of the resulting extract was used in 3 cc. of reaction mixture, except for leucylglycine and leucyldiglycine (0.26 cc.).

‡ Relative activities in the presence of MnSO<sub>4</sub>.

sulting preparation has a specificity like that of hog leucylpeptidase. The hydrolyses of all six peptides by the leucylpeptidase are activated by both Mg and Mn ions.

From the data of Table VIII, a more detailed comparison may be made of the specificities of hog and *Pseudomonas* leucylpeptidases. In most instances the peptides and their substituted derivatives were hydrolyzed by the bacterial leucylpeptidase at practically the same rates as by hog and plant leucylpeptida-



ses (6). However, the bacterial enzyme resembled the plant leucylpeptidases rather than the hog or chick leucylpeptidase in its slower rate of hydrolysis of glycyl-*dl*-leucylglycine.

Preliminary data obtained with the bacterium *Rhizobium trifolii*, Strain 209, suggest the presence of a leucylpeptidase in this organism. As shown in Table IX, the hydrolyses of both

TABLE VIII  
*Specificities of Leucylpeptidases of Pseudomonas fluorescens and Hog Intestine*

Substrate	<i>Pseudomonas fluorescens</i> *			Hog†		
	Per cent hydrolysis of one linkage in					
	1 hr.	5 hrs.	24 hrs.	1 hr.	5 hrs.	24 hrs.
<i>dl</i> -Leucyldiglycine.....	32	66	86	39	101	110
<i>dl</i> -N-Methylleucyldiglycine.....	0	4	10	0	0	5
Glycyl- <i>dl</i> -leucylglycine‡.....	14	70	190	76	194	206
<i>dl</i> -Alanyldiglycine.....	2	16	52	10	53	112
Triglycine.....	0	4	14	0	3	12
Sarcosyldiglycine.....	0	2	3	0	0	2
Tetraglycine.....	0	4	8	0	2	6
<i>dl</i> -Leucylglycine.....	20	96	100	40	100	105
<i>dl</i> -Leucylmethylamine.....	0	2	2	6	10	17
Diglycine.....	2	4	8	1	2	13
Glycylmethylamine.....	0	0	2	0	0	-1
<i>dl</i> -N-Methylleucylglycine.....	1	2	10	0	4	21
<i>dl</i> -Alanylglycine.....	6	16	58			
<i>dl</i> -Prolylglycine.....	0	2	8	0	5	24
<i>dl</i> -Prolyldiglycine.....	0	0	16	-1	4	23
<i>dl</i> -Leucyldiglycine + 0.001 M MnSO <sub>4</sub> .....	68	100	110			

\* 0.3 cc. of an acetone-precipitated preparation was used in 3 cc. of reaction mixture.  $m/300$  MgCl<sub>2</sub> was present in all cases except the last.

† The data are taken from Table VIII of a previous paper (6).

‡ The sparingly soluble glycyl-*dl*-leucylglycine was present in  $m/30$  concentration.

leucylglycine and leucyldiglycine are activated by 0.003 M MgCl<sub>2</sub>. pH-activity determinations with a crude extract indicated a maximum rate of leucylglycine hydrolysis at pH 7.8 and of leucyldiglycine hydrolysis at pH 8.6, as shown in Fig. 4. The difficulty of obtaining sufficiently active preparations prevented the more detailed investigation of this proteolytic system.

It may be mentioned here that the leucylpeptidase of *Phytomonas tumefaciens* which was previously reported to be activated

TABLE IX  
*Peptidases of Rhizobium trifolii*

Substrate	Time of incubation	Hydrolysis*	
		No activator	0.003 M MgCl <sub>2</sub>
	hrs.	per cent	per cent
dl-Alanylglycine.....	5		66
dl-Leucylglycine.....	7.5	56	70
Diglycine.....	5		24
dl-Alanyldiglycine.....	10		28
dl-Leucyldiglycine.....	10	40	64
Triglycine.....	10		22

\* 1 cc. of crude extract (equivalent to 150 mg. of wet cells) was used in 3 cc. of reaction mixture.

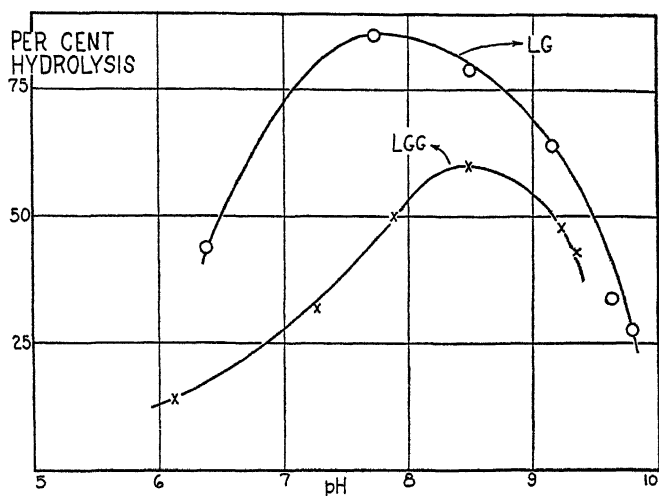


FIG. 4. pH optima for the hydrolysis of leucylglycine (LG) (8 hours incubation period) and leucyldiglycine (LGG) (9 hours incubation) by a crude extract of *Rhizobium trifolii*, Strain 209. All determinations were made with the same amount of enzyme solution in the presence of 0.003 M MgCl<sub>2</sub>.

by Mg ions (7) is also activated to an even greater extent by 0.001 M Mn ions.

Leucylpeptidases have been found in several tissues of the lobster, *Homarus americanus*. As shown in Table X, leucylglycine and leucyldiglycine hydrolyses by dialyzed enzyme preparations from the hepatopancreas, stomach, intestine, and abdominal muscle are activated by Mg and Mn ions, although in some cases

TABLE X  
*Peptidases of Lobster Tissues*

Source*	<i>dl</i> -Leucylglycine				<i>dl</i> -Leucyldiglycine			
	Time of incubation	Hydrolysis			Time of incubation	Hydrolysis		
		No activator	0.001 M MnSO <sub>4</sub>	0.01 M MgSO <sub>4</sub>		No activator	0.001 M MnSO <sub>4</sub>	0.01 M MgSO <sub>4</sub>
	hrs.	per cent	per cent	per cent	hrs.	per cent	per cent	per cent
Stomach.....	2	24	54	28	4	16	68	25
Hepatopancreas.....	1	40	92	50	1	15	58	26
Muscle.....	4	41	70	44	5	16	54	24
Intestine.....	6	13	48	28	6	4	28	14

\* Extracts were dialyzed against distilled water for 24 hours at 2° before analysis. The same amount of enzyme was used in each pair of determinations.

TABLE XI  
*Peptidases of Cockroach Intestine*

Substrate	Time of incubation	Hydrolysis*			
		No activator	0.01 M MgSO <sub>4</sub>	0.001 M MnSO <sub>4</sub>	0.001 M CoCl <sub>2</sub>
	hrs.	per cent	per cent	per cent	per cent
<i>dl</i> -Leucylglycine.....	7.5	15	26	56	
<i>dl</i> -Leucyldiglycine.....	7.5	12	24	40	
Diglycine.....	8	10	11	20	16

\* A crude extract dialyzed for 25 hours at 2° was used as enzyme source. The same amount of enzyme was used in all the determinations.

the Mg activation is very small indeed, a property previously observed only with certain malt and fish leucylpeptidase preparations.

The existence of a leucylpeptidase-like enzyme in the intestine of the cockroach, *Periplaneta australasiae*, is shown in Table XI. It may be seen that the hydrolysis rates of both leucylglycine

and leucyldiglycine were markedly increased by Mg and Mn ions. The presence of an Mn-Co-activated dipeptidase, described previously (3), is also indicated.

Some organisms, for example yeast and molds, do not appear to possess a leucylpeptidase. Autolysates of brewers' bottom yeast were analyzed after 2 hours and after 7 days of toluene autolysis at pH 6.5. Determinations were made on six peptides in the presence and absence of Mg and Mn salts but in no case was there any indication of the presence of a leucylpeptidase.

Neither Mg nor Mn ions activated leucyldiglycine hydrolysis by crude or purified tissue extracts of the mold *Aspergillus parasiticus* and 0.001 M Mn ions slightly inhibited the hydrolysis of leucyldiglycine by tissue extracts from the molds *Penicillium terrestre* and *Penicillium citrinum*. These facts strongly suggest the absence of a leucylpeptidase type of enzyme in these molds.

#### SUMMARY

1. Relatively pure leucylpeptidase preparations may be conveniently prepared from a number of sources by precipitation of crude extracts at pH 6 to 7 with 1 volume of acetone. The water-soluble portion of the resulting precipitate contains the enzyme.

2. Leucylpeptidases are shown to be present in the proteolytic systems of human duodenum, rat intestine, rat carcinoma tissue, chick intestine, trout tissues, lobster tissues, cockroach intestine, and in the bacteria *Pseudomonas fluorescens* and *Rhizobium trifolii*. The characteristics which were used to identify the enzyme were (a) activation by Mg and Mn ions, (b) an approximately equal rate of hydrolysis of leucylglycine and leucyldiglycine, (c) rapid hydrolysis of leucylglycine and leucyldiglycine, slow hydrolysis of alanylglycine and alanyldiglycine, and very slow hydrolysis of diglycine and triglycine, (d) a pH optimum of 8 to 9.5.

3. Leucylpeptidases were not found in brewers' yeast or in the molds *Aspergillus parasiticus*, *Penicillium citrinum*, and *Penicillium terrestre*.

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## STUDIES ON KETOSIS

### •XVII. THE RATE OF DISAPPEARANCE OF $\beta$ -HYDROXY-BUTYRIC ACID IN FASTED AND FED RATS\*

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The mechanism by which administered glucose is able to decrease the level of ketonuria in fasting animals has been explained by widely divergent theories. On the one hand the idea has been widely accepted that glucose or its intermediary products are able chemically to unite with or in some way catalyze the oxidation of the acetone bodies by a process which may be referred to as ketolysis. Opposed to this is the suggestion that glucose is preferentially oxidized when so administered, thus sparing a certain amount of fat breakdown with its resultant production of ketone bodies. The latter theory ascribes the reduction in ketonuria to an antiketogenic effect of carbohydrate.

There are several strong arguments which would seem to point to the first explanation as the more acceptable one. The *in vitro* experiments of Shaffer (1) demonstrate the marked catalytic power of glucose in causing the oxidation of acetoacetic acid. Moreover, it has been shown by Shapiro (2) that the feeding of any glucose precursor diminishes the ketone body elimination resulting when acetoacetic acid is fed, although isodynamic quantities of non-glucose formers are entirely ineffective in this respect. Since the acetoacetic acid could not be stored, the most probable explanation for the decreased excretion in the urine is that the administered metabolite has catalyzed its oxidation. The magnitude of decrease in ketone body elimination is far in excess of what

\* This work was assisted by a research grant from the Rockefeller Foundation.

may be excreted from endogenous sources even under such favorable conditions for its production as occur in fasting rats which have fatty livers. Later, Deuel, Hallman, and Murray (3) demonstrated that the exogenous ketonuria of rats receiving sodium butyrate as well as the endogenous ketonuria resulting after the administration of a high fat diet could be largely abolished by an amount of glucose equal in energy value to less than 1 per cent of the total fat catabolism. Isodynamic amounts of alcohol were entirely ineffective in altering the ketonuria.

On the other hand, Mirsky and Broh-Kahn (4) were unable to show any alteration in the rate of disappearance of injected  $\beta$ -hydroxybutyric acid in rabbits receiving glucose from that of fasted animals. These investigators concluded that glucose must decrease ketonuria by an antiketogenic action. More recently Mirsky, Nelson, and Grayman (5) have reported that no difference in the rate of oxidation of injected  $\beta$ -hydroxybutyric acid could be noted in well fed and fasted nephrectomized rats as judged by the unmetabolized ketones recovered from the carcass.

In the present study the rate of disappearance of *l*- $\beta$ -hydroxybutyric acid has been followed in fasted and well fed nephrectomized rats for 75 and 150 minutes. So that neither the effect of the trauma nor that of ether anesthesia might be a factor, the operations were performed approximately 20 hours before the tests were carried out.

#### *Procedure*

The experiments were performed on female rats from our stock colony, approximately 3 months of age. The body weights used for calculation of the doses of  $\beta$ -hydroxybutyric acid in all cases were those obtained 2 days before the experiments, at the start of the fast for the inanition group. Nephrectomy was carried out under ether anesthesia between 1 and 3 p.m. on the day previous to the tests. In order to determine the food consumption of the fed group, the animals were placed in separate cages following nephrectomy and their food intake determined until the start of the experiments. At that time they were given 0.5 cc. of 50 per cent glucose per 100 sq. cm. of body surface by stomach tube. The fasted rats received orally a similar dose of 8 per cent sodium chloride solution which is isotonic with the glucose. In the experi-

ments in which  $\beta$ -hydroxybutyric acid was administered, it was injected intraperitoneally 30 minutes after the glucose or salt solution was administered. *l*- $\beta$ -Hydroxybutyric acid was given in doses equivalent to 75 mg. per 100 sq.cm. as acetone or 143 mg. calculated as the acid. The quantities of solution of this acid injected were approximately 1.00 cc. in our tests. This dose is the level that Butts and Deuel (6) have employed in each of two daily feedings for the establishment of an exogenous ketonuria. It is too great to administer intravenously in a short period but it is readily tolerated when given by mouth or intraperitoneally. In fact, except for the slight discomfort which our animals exhibited for a few minutes after injection owing to the hypertonic solution, they remained in apparently normal condition to the termination of the experiment. We have lost only two experiments out of forty-six carried out owing to the death of the rats, while two others were discarded because diarrhea developed. *l*- $\beta$ -Hydroxybutyric acid was prepared from rat urine by the procedure of Blunden (7). After purification as the calcium zinc salt,<sup>1</sup> the free acid was separated by ether extraction. The latter was partially neutralized with sodium hydroxide to pH 7.4.

The rats were killed 75 and 150 minutes after the  $\beta$ -hydroxybutyrate had been injected. The control rats were killed after the same interval had elapsed, following glucose or sodium chloride solutions, as the animals which were given the  $\beta$ -hydroxybutyrate. Amytal was used for an anesthetic. The rat was ground by passing it through a meat chopper three times and the hashed material was used for the determination of the acetoacetate and acetone and also the  $\beta$ -hydroxybutyrate fractions.

Acetoacetate and acetone were determined by distillation from a weighed aliquot (usually 50 gm.) in a distilling flask with 250 cc. of water and 20 cc. of 50 per cent sulfuric acid. The process was continued until 120 cc. of distillate were collected in the receiving flask which was immersed in an ice bath. An appropriate amount of the distillate was then used without further treatment for the determination of acetone by the Van Slyke technique. No acetone

<sup>1</sup> Two samples of the calcium-zinc salt were used for making the sodium *l*- $\beta$ -hydroxybutyrate. One sample was prepared by one of us (L. H.) and the other by B. Bobbitt. Both showed the theoretical optical rotation for *l*- $\beta$ -hydroxybutyrate.



originates from  $\beta$ -hydroxybutyrate when subjected to this procedure. When known amounts of diacetic acid were injected intraperitoneally and the rats immediately killed, an average of 79.7 per cent was recovered. We have therefore corrected our analyses by this value.

For the determination of  $\beta$ -hydroxybutyrate, an aliquot of 50 gm. of hashed tissue was mixed with 125 cc. of water and the whole brought to a boil with constant stirring. The fluid was expressed with a potato ricer, and the practically dry cake remaining was reextracted with 75 cc. of water by a similar procedure. This was repeated a third time with 75 cc. To the combined extracts in a stoppered graduated cylinder, 10 cc. of 50 per cent zinc sulfate solution and 2 cc. of 40 per cent sodium hydroxide were added and the volume noted. After thorough mixing, the protein was filtered off and the filtrate subjected to the copper lime precipitation.  $\beta$ -Hydroxybutyrate determinations were made on the filtrate by the Van Slyke procedure. When known amounts of  $\beta$ -hydroxybutyrate were injected intraperitoneally into well fed rats, and the animals immediately sacrificed, the carcasses ground, and 50 gm. aliquots extracted, an average of 109.9 per cent of the  $\beta$ -hydroxybutyrate was recovered in eleven determinations after allowance was made for the ketone body content of tissues already present. The higher than theoretical recovery is probably caused by the large amount of precipitate due to which the  $\beta$ -hydroxybutyrate was not distributed uniformly throughout. On the other hand when we have used only 25 gm. of tissue, the recovery in three experiments averaged 100.6 per cent. However, because we have always used 50 gm. samples, the values for  $\beta$ -hydroxybutyrate recovered have been corrected. The agreement between different aliquots of the same tissue is quite satisfactory.

### *Results*

A summary of the experimental data is given in Table I. This includes the values for the acetone bodies in the tissues of control rats which received no  $\beta$ -hydroxybutyric acid, but which were killed at comparable periods to the experimental animals. Table II gives the results of the statistical treatment of the experimental data.

TABLE I  
Utilization of *l*- $\beta$ -Hydroxybutyrate by Fasted and Fed Nephrectomized Female Rats

Group No. and nutritional condition	Period after in- jection before killing	No. of ex- peri- ments	Body weight*	Surface area*	Food eaten after nephrec- tomy	Acetone bodies as acetone in mg.								
						<i>l</i> - $\beta$ -Hydroxy- butyrate in- jected	Recovered			Utilized per 60 min.†				
							Acetoacetate	$\beta$ -Hydroxy- butyrate	Total	Corrected total	Per 100 gm.			
											(1)	(2)		
<i>l</i> - $\beta$ -Hydroxybutyrate injected														
I. Fasted.....	75	12	gm. 140 (-10)	sq. cm. 243 (-11)	gm. 1.3 (7)	185.8	26.8	146.6	173.4	149.0	20.9 ±2.1	21.7 ±2.2	12.1 ±1.2	12.6 ±1.2
II. Fed.....	75	11	gm. 144 (+1)	246 (0)		187.8	13.5	108.0	121.5	118.3	38.6 ±2.6	38.6 ±2.1	22.5 ±1.5	22.5 ±1.5
III. Fasted.....	150	10	gm. 147 (-11)	250 (-11)		190.8	30.8	87.9	118.7	93.6	26.4 ±2.2	28.3 ±2.4	15.4 ±1.3	16.1 ±1.3
IV. Fed.....	150	9	gm. 144 (+4)	246 (+5)		187.9	10.3	37.5	47.8	44.7	40.0 ±1.0	38.9 ±1.3	23.2 ±0.4	22.8 ±0.5
No <i>l</i> - $\beta$ -hydroxybutyrate administered														
						Acetone bodies in tissues								
V. Fasted.....		11	144 (-12)	247 (-12)			4.9	20.2	25.2		17.1 ±1.0	18.7 ±1.1	10.0 ±2.3	10.6 ±2.4
VI. Fed.....		12	151 (-1)	254 (0)	3.3 (10)		0.2	3.1	3.3		2.12 ±0.18	2.17 ±0.18	1.26 ±0.36	1.28 ±0.33

\* Body weight and surface area 2 days before the animals were killed. Values in parentheses indicate the change in those values at the time of killing.

† Including the standard error of the mean,  $s.e.m. = \sqrt{d^2/n} / \sqrt{n}$ . (1) Calculations on the basis of original weight or surface area and (2) on these values at the time the animals were killed.

‡ One atypical experiment excluded in which unusually high retention of fluid was noted.

## DISCUSSION

When *l*- $\beta$ -hydroxybutyric acid, partially neutralized to pH 7.4, is injected into fasting rats, comparatively large amounts disappear. Presumably the disappearance is best explained as a result of oxidation, since there seems to be evidence that the ketone bodies are not convertible to neutral fat or to carbohydrate which might be stored. When the rats had considerable stores of carbohydrate available supplemented by the oral administration of 50 per cent glucose solution 30 minutes before the acid was given, the rate of removal of the hydroxy acid was considerably increased. The variation was somewhat greater when the concen-

TABLE II  
*Statistical Evaluation of Differences in Utilization Rate*

Groups compared	Weight basis of comparison	Ratio, mean difference to standard error of difference*		Per cent of fed rats above control level	
		On weight basis	On surface area basis	On weight basis	On surface area basis
75 min., Groups I and II	Original	5.32	5.42	100	100
	Killing	5.54	5.10	100	100
150 " " III " IV	Original	5.12	5.82	100	100
	Killing	3.95	4.96	100	100

\* Standard error of difference =  $\sqrt{(\text{S.E.M.}_1)^2 + (\text{S.E.M.}_2)^2}$ .

tration was highest (in the 75 minute group) but was still evident in the experiments carried on for a longer interval.

We have felt that the most satisfactory basis for comparison of the utilization is on the normal weight prior to the initiation of fasting. The lowering in body weight due to a 48 hour fast does not lower the active metabolizable tissue by a corresponding amount. However, we have carried out our calculations of the rate of utilization also on the weight just prior to killing the animals and the results are similar. Calculations are also included on the rates of utilization based on surface area, which are probably a better index than body weight for comparison. An average of only 12.1 mg. of  $\beta$ -hydroxybutyrate (as acetone) was utilized per 100 sq.cm. per hour during the 75 minute period following the injection of 75

mg. of this substance in fasting rats; in those rats which were fed, the average utilization was 22.5 mg. (as acetone) per hour. In the 150 minute period, the rates of utilization were approximately the same, being 15.4 and 23.2 mg. per hour for the fasted and fed groups respectively. When the surface area is calculated on the killing weight, the corresponding rates of utilization for the fasted and fed rats for the shorter period are 12.6 and 22.5 mg. per hour respectively; for the longer interval the corresponding averages are 16.1 and 22.8 mg. According to the statistical treatment given in Table II, it is noted that the differences in rate of utilization between the fasted and fed groups at both intervals are statistically significant when the surface area is based either on the original weight or on the weight at the time the animals were sacrificed.

These results are at variance with the report of Mirsky, Nelson, and Grayman (5) who have found no statistically significant differences in the rats subjected to inanition as compared with the glucose-fed animals. There are several explanations for their failure to observe such differences. One of the most serious of these is the fact that the studies were carried out on rats immediately after recovery from ether anesthesia following nephrectomy. The depressing effect of ether on carbohydrate metabolism is well known and one would not expect a normal response during a period immediately following its administration. The trauma attendant on the operation might also interfere with a normal response. To avoid any such complications, nephrectomies were performed on the day prior to the test in the present study.

A more serious objection to the experiments of Mirsky, Nelson, and Grayman (5) is the fact that the quantity of  $\beta$ -hydroxybutyric acid administered was too small. Butts and Deuel (6) have shown that no consistent ketonuria is obtained in fasting rats when quantities of ketone bodies less than 75 mg. per 100 sq.cm. (as acetone) are administered twice daily. It is only when the amount of ketogenic acid fed exceeds the ability of the tissues to destroy it that an appreciable ketonuria occurs. However, when such a ketonuria is produced, it may be decreased when glucose is fed to the rat; this fact must indicate that a more rapid utilization has occurred in the tissues with a smaller resultant excess to be excreted.

The maximum amount of  $\beta$ -hydroxybutyric acid administered by Mirsky, Nelson, and Grayman was only 8.40 mm per kilo, which is equivalent to 51.6 mg. as  $\beta$ -hydroxybutyric acid or 28.8 mg. as acetone per 100 sq.cm. This is slightly over one-third the dose employed by us of 75 mg. as acetone per 100 sq.cm. The rate of utilization of  $\beta$ -hydroxybutyric acid, namely 42.8 and 41.7 mg. as acetone per hour, found by the above investigators in their fasting rats is much higher than the averages noted by us in our corresponding groups which were 20.9 and 26.4 mg. respectively.

Although any experimental procedure which involves the administration of large amounts of even a usual metabolite must be considered abnormal, the administration by the intravenous route affords a more severe shock than that by the intraperitoneal pathway, which allows the gradual distribution to the tissues. This is particularly the case when the substance concerned causes an upset in the acid-base equilibrium. That the shock of intravenous injection was far greater in Mirsky's tests<sup>2</sup> than in our own, in which the intraperitoneal pathway was employed, is evidenced by the fact that a considerable mortality obtained in the former case with only a 40 minute interval elapsing, while only two fatalities occurred in our tests which were prolonged up to  $2\frac{1}{2}$  hours. The purification of the  $\beta$ -hydroxybutyric acid by ether extraction followed by the neutralization to pH 7.40 may also account for the better tolerances observed in the present series. Not only have we found that unpurified commercial sodium  $\beta$ -hydroxybutyrate may contain appreciable amounts of excess base, but also on the basis of theoretical considerations the calculated pH of a 2.6 M solution of the pure salt is 9.56.<sup>3</sup>

Another difference in technique employed in this series of experiments as compared with those of Mirsky *et al.* (5) is that we have used the active isomer rather than the racemic  $\beta$ -hydroxybutyrate. However, it seems probable that the variations reported here are not ascribable to that fact. In an earlier more extensive series of unpublished tests, it was also found that there were differences in the rate of utilization of purified *dl*- $\beta$ -hydroxybutyrate in fasted and fed rats. The average utilization of *dl*- $\beta$ -hydroxybutyrate

<sup>2</sup> Personal communication.

<sup>3</sup> Calculated from the following formula:  $\text{pH} = \frac{1}{2}\text{pK}_w + \frac{1}{2}\text{pK}_a + \frac{1}{2}\log C$ .  $K_w = 2.0 \times 10^{-14}$ .  $C$  is the concentration of the  $\beta$ -hydroxybutyrate.

(calculated as mg. of acetone per 100 sq.cm. per hour) in fasted rats was 24.2, 17.3, and 17.6 mg. respectively in tests lasting 75, 120, and 150 minutes; on the other hand the corresponding rates found for fed rats were 32.8, 23.7, and 22.0 mg. respectively. Subsequently it was noted that the method of recovery employed in this earlier series, although satisfactory for the hydroxybutyrate, failed to account for the acetoacetate fraction. However, in the present series it has been noted that the differences in the acetoacetate fraction in the tissues of fasted and fed rats are much more pronounced than in the  $\beta$ -hydroxybutyrate fraction. Had the acetoacetate been satisfactorily recovered in the above tests, it appears probable that the variations between the fasted and fed groups would have been even greater than the figures given above would seem to indicate.

One may suggest that the more rapid disappearance of the ketone bodies in the glucose-fed rats is due to the suppression of formation of new  $\beta$ -hydroxybutyric acid by the antiketogenic action of the sugar. On the other hand, in the fasted rats the ketone bodies continue to be produced in endogenous metabolism; therefore, the total  $\beta$ -hydroxybutyric acid isolated from the carcass is increased by that formed from endogenous sources. However, to obtain the amount utilized, correction is made for the amount which would have been present had no  $\beta$ -hydroxybutyric acid been given. Although this quantity does not represent the total turnover of  $\beta$ -hydroxybutyric acid, it does represent the amount present at any time resulting from the equilibrium between that produced and oxidized by endogenous processes.

The most logical conclusion from these experiments seems to be that glucose increases the rate of disappearance of  $\beta$ -hydroxybutyric acid from the tissues of the fasting rat provided a sufficient dose is given so that there is an excess in the tissues. The greater rate probably is not due to a suppression of formation of this compound from endogenous sources (antiketogenesis) but is more easily explained by an acceleration in oxidation caused by glucose (ketolysis).

#### SUMMARY

A procedure is described for recovery of  $\beta$ -hydroxybutyric and acetoacetic acids from the hashed tissues of the rat.

When *L*- $\beta$ -hydroxybutyrate was injected intraperitoneally into fasted, nephrectomized rats, the rate of disappearance was found to be significantly lower in experiments carried on for 75 and 150 minute intervals than was noted for animals receiving glucose.

It is concluded that the rate of disappearance of  $\beta$ -hydroxybutyrate from the tissues is accelerated when glucose is present. The decrease in ketonuria noted by us earlier following the administration of carbohydrate would seem to be traced to an increased utilization of the ketone bodies in the tissues.

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## THE EFFECT OF MALONATE ON TISSUE RESPIRATION\*

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Malonate has long been regarded as a specific inhibitor for succinic dehydrogenase. The original evidence was obtained in Thunberg experiments with resting bacterial cultures (1, 2), and similar results were later obtained manometrically with brain and muscle tissue (3). The apparent specificity of this inhibition has led workers to use respiration experiments with malonate as a basis for theories of hydrogen transport (4, 5) and of carbohydrate breakdown—the “citric acid cycle” of Krebs and Johnson (6)—inasmuch as malonate inhibits the respiration of intact tissue as well as of enzyme preparations. However, analytical data of Weil-Malherbe (7) suggest that malonate may not act specifically on succinic dehydrogenase in intact tissue, even though its action on purified enzyme systems be highly specific. Moreover Das (8) and Szent-Györgyi (9) have pointed out that fumarate and succinate differ quantitatively rather than qualitatively in their action on malonate-poisoned enzyme systems, and preliminary data of our own also seemed to indicate that malonate might not be a specific inhibitor (10). The experimental basis for the citric acid cycle has therefore been reexamined by a study of the respiration of the various component acids in the presence of malonate.

### EXPERIMENTAL

All experiments were performed with minced pigeon breast muscle. The bird was decapitated, and the muscle removed as rapidly as possible, chilled on ice for 2 to 3 minutes, and minced

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in a chilled Latapie mincer. The mince was collected in a chilled Petri dish lined with filter paper moistened with saline solution. The tissue samples were weighed on cellophane on a torsion balance and the material dispersed in the appropriate solution contained in manometric flasks by means of a wire.

The oxygen consumption of the tissues was measured at 38° in the usual way in a standard Warburg apparatus. Unless otherwise stated, the medium used was a Ringer-phosphate buffer free of calcium, pH 7.4, in which muscle is known to be more sensitive to the dicarboxylic acids (11). The buffer was made up in twice normal concentration and diluted either with water or with supplementary solutions.

The following acids were studied for their effect upon muscle respiration in the presence and absence of malonate: citric,  $\alpha$ -ketoglutaric, succinic, fumaric, and malic. In addition *l*(+)-glutamic acid was studied, since by oxidative deamination, it yields  $\alpha$ -ketoglutaric acid (12, 13). The salts and acids were all commercial preparations except  $\alpha$ -ketoglutaric acid.<sup>1</sup> The solutions of the acids or their sodium salts were neutralized at concentrations of 0.2 M, and further dilutions then made from the neutral solution. Fresh solutions were made up at weekly intervals and stored at 0° when not in use. Solutions of  $\alpha$ -ketoglutaric acid, however, were always made up just prior to their use.

The results of the manometric experiments were expressed in c.mm. of O<sub>2</sub> absorbed per mg. of tissue (dry weight) (11). Most experiments were run for 2 hours. In the analysis of results each experimental observation was compared with its own control, since the respiration of pigeon muscle varies from bird to bird. While for convenience, only the averages of many experiments have been tabulated, the results themselves were remarkably consistent. 0.001 or 0.005 M malonate invariably inhibited respiration as compared to the control, and the other acids invariably increased it. A similar consistency was observed when mixtures of acids were studied. Respiration was always better in fumarate plus malonate than in equimolar succinate plus malonate, which in turn was always better than citrate plus malonate.

We are indebted to M. A. Lipton for this preparation.

*Results*

While malonate inhibited muscle respiration in concentrations of 0.001 M or less, the most suitable concentration for a comparison of the sensitivity of the various acids to malonate was found to be 0.005 M. This concentration of malonate inhibited respiration about 70 per cent, and the various acids studied were markedly unequal in their ability to prevent this inhibition. Two levels of respiration were used for comparison, (a) the oxygen consumed by the unsupplemented control sample, and (b) that consumed by tissues supplemented with the various acids in concentrations of 0.001 or 0.005 M. This latter was designated "stabilized respiration." Over a 2 hour period glutamic acid increased respiration nearly 50 per cent; the other acids increased respiration about 30 per cent.

All of the acids studied stimulated respiration catalytically; that is, the extra oxygen consumed in the presence of small amounts of the acid was greater than the amount required completely to oxidize the added acid. Such data have already been reported for fumaric, malic, succinic (11), citric, and  $\alpha$ -ketoglutaric acids (6, 14). We have been able to confirm the results of Krebs and Johnson with regard to the latter two acids, using Ringer-phosphate buffer, and in addition have observed catalysis with *l*(+)-glutamic acid. The amount of oxygen needed completely to oxidize 2 cc. of 0.0004 M glutamic acid is 80.6 c.mm. The extra oxygen consumed by 33.2 mg. (dry weight) of pigeon muscle in the presence of the acid was 249 c.mm. over a 2 hour period.

In Table I the various acids are listed in decreasing order of effectiveness in counteracting malonate inhibition. Fumarate and malate were most effective. In the presence of 0.005 M malonate, 0.005 M fumarate or malate restored respiration not only to the level of the unsupplemented control, but also to that of "stabilized respiration." When only half as much fumarate or malate was used, 0.0025 mole, the respiration was slightly below that of the control sample.

$\alpha$ -Ketoglutarate was nearly as effective as fumarate or malate. In equimolecular amounts of malonate and  $\alpha$ -ketoglutarate, respiration exceeded that of the unsupplemented control, although it did not reach that of the "stabilized" samples.

Succinate was less effective than  $\alpha$ -ketoglutarate in compensating for malonate inhibition. In equimolecular amounts of succinate and malonate, respiration was slightly less than that of the unsupplemented control, but when twice as much succinate as malonate was used, respiration was restored to the "stabilized" level.

TABLE I

*Respiration of Pigeon Breast Muscle in Presence of 0.005 Mole of Malonate and Various Stabilizing Supplements*

All data are expressed as c.mm. of  $O_2$  absorbed per mg. of tissue (dry weight) in 2 hours.

Acid supplements	Respiration in presence of supplement		Control respiration, unsupplemented		No. of experiments
	Malonate	No malonate	Malonate	No malonate	
0.0025 M malic.....	11.7	14.3	3.8	13.1	2
0.005 " ".....	15.1	14.3	3.8	13.1	2
0.0025 " fumaric.....	13.4	17.0	3.9	14.9	3
0.005 " ".....	18.6	18.7	4.3	13.4	3
0.01 " ".....	20.5	21.7	6.2	19.1	2
0.0025 " $\alpha$ -ketoglutaric.....	12.4	18.3	3.8	13.1	2
0.005 " ".....	16.7	20.3	3.3	15.8	5
0.01 " ".....	15.6	18.3	3.5	13.8	3
0.005 " succinic.....	13.3	19.8	4.4	16.2	8
0.01 " ".....	19.6	19.8	4.4	16.2	8
0.005 " glutamic.....	10.4	24.4	3.9	17.4	3
0.01 " ".....	14.0	22.7	3.9	17.4	3
0.005 " citric.....	8.3	19.8	4.4	16.2	8
0.01 " ".....	8.0	19.8	4.7	16.4	7

Glutamic acid and citric acid were the least effective acids in counteracting malonate inhibition. These acids failed to restore respiration to the control level even in concentrations twice or 5 times<sup>2</sup> that of the malonate used. When 0.001 M malonate, and corresponding amounts of the various supplementary acids were used, the differences observed between acids were less marked than in the presence of the higher level of

<sup>2</sup> This latter result should not be stressed, since citrate alone frequently inhibits respiration in concentrations above 0.02 M. However, at the critical concentration 0.01 M, citrate alone stimulated respiration.

malonate. However, citrate was again the least effective of all in counteracting malonate inhibition.

TABLE II

*Effect of Di(Tri)-Carboxylic Acids and Malonate on Respiration of Supplemented Pigeon Breast Muscle*

All values are expressed as c.mm. of O<sub>2</sub> absorbed per mg. of tissue (dry weight) in 4 hours.

Supplements	Respiration		Per cent inhibition
	Malonate*	No malonate	
0.005 M malate + muscle juice + insulin.....	30.3	41.9	28
0.01 " " + " " + " .....	36.0	44.0	18
0.005 " " + " " + " + cocarboxylase.....	39.0	37.7	0
0.005 M fumarate + muscle juice + insulin....	29.8	38.2	22
0.001 " " + " " + " ....	29.0†	30.1	3.6
0.005 " α-ketoglutarate + muscle juice + insulin.....	24.0	31.2	23
0.01 M α-ketoglutarate + muscle juice + insulin.....	27.3	24.8‡	0
0.001 M α-ketoglutarate + muscle juice + insulin.....	9.4†	11.2	16
0.005 M succinate + muscle juice + insulin....	16.7	29.7	43
0.01 " " + " " + " ....	29.1	34.0§	14.2
0.005 " " + " " + " + cocarboxylase.....	20.3	38.8	48
0.005 M succinate + muscle juice + insulin....	21.8	34.4	37
0.005 " glutamate + " " + " ....	12.3	40.9	70
0.005 " citrate + muscle juice + insulin.....	8.3	29.2	71
0.01 " " + " " + " .....	9.6	26.5	64
0.001 " " + " " + " .....	12.2†	15.0	19

\* The malonate concentration is 0.005 M, except where 0.001 M was used, as indicated by †.

‡ 0.005 M α-ketoglutarate + muscle juice + insulin.

§ 0.005 M succinate + muscle juice + insulin.

|| 0.005 M citrate, etc.

The differences between the effects of the various acids persisted in the presence of other supplements, as for example, combinations of muscle juice (*Kochsafft*) (11), insulin, and cocarboxylase. The respiration of tissue thus highly fortified was

decreased 28 per cent or less in the presence of equimolecular amounts of malonate and fumarate, malonate and malate, or malonate and  $\alpha$ -ketoglutarate (Table II). In the presence of equimolecular amounts of malonate and succinate "fortified" respiration was decreased 40 per cent but, as in "unfortified" tissue, respiration was increased when the amount of succinate was doubled. In fortified tissue 0.005 M malonate decreased respiration 70 per cent in the presence of equimolar citrate or

TABLE III

*Increments of Oxygen Uptake during Various Periods of Respiration Following Delayed Addition of Supplements*

All data are expressed as c.mm. of  $O_2$  absorbed per mg. of tissue (dry weight).

Supplements (0.005 M)	1st 30 min.	2nd 30 min.	3rd 30 min.	4th 30 min.
Control.....	4.8	3.3	1.9	1.2
Malonate at 0 min.....	1.9	0.8	0.2	0.2
"    " 30 " .....	5.2	1.2	0.4	0.3
Citrate " 0 " malonate at 0 min.....	2.5	1.1	0.4	0.2
"    " 0 " " " 30 " .....	6.0	1.9	0.7	0.4
Fumarate " 0 " " " 0 " .....	5.0	3.1	2.1	1.2
"    " 0 " " " 30 " .....	5.3	2.9	2.0	1.3
Succinate " 0 " " " 0 " .....	4.8	3.9	2.8	1.7
"    " 0 " " " 30 " .....	8.7	5.0	2.8	2.0
$\alpha$ -Ketoglutarate at 0 min., malonate at 0 min.	4.8	3.6	2.6	1.8
"    " 0 " , " " 30 " .....	6.2	5.4	4.4	3.5
Malonate at 0 min., fumarate at 30 min.....	2.2	3.0	2.1	1.5
"    " 0 " succinate " 30 " .....	2.1	2.6	2.2	1.4
"    " 0 " $\alpha$ -ketoglutarate at 30 min.	2.4	2.1	1.2	0.6
"    " 0 " citrate at 30 min.....	2.0	1.3	0.6	0.7

glutamate. Since malonate inhibition ranged from 60 to 80 per cent in tissues variously fortified, it is doubtful whether the citrate or glutamate had exerted any protective action at all. Increased amounts of citrate failed to decrease the inhibition materially.

Malonate was also added to media in which tissues had been respiring for 30 minutes in the presence of the various acids, thus allowing time for the conversion of the acids into other active components as postulated in the citric acid cycle of Krebs and Johnson (6). Under these circumstances the effect of the in-

hibitor might have been modified. Actually, however, the same differences between the various acids appeared as before (Table III). Citrate was again the least effective acid in compensating for malonate inhibition. When malonate was added first, followed 30 minutes later by the various acids, fumarate and succinate increased the respiratory rate over that of the previous period, whereas citrate failed to halt the rapid decline in the rate of respiration (Table III).

#### DISCUSSION

The differences in the activity of the various acids in the presence of malonate were apparently not due to differences in their rates of penetration into the cell, since in the absence of malonate the various acids all stimulated respiration to approximately the same degree. Such differences as were observed could not be correlated with malonate sensitivity, although a survey of a large variety of tissues supplemented in various ways indicated that citrate catalysis was less frequent than fumarate catalysis (10). Glutamate, however, was the most effective acid of all in stimulating respiration, although, like citrate, its effect was completely nullified by malonate.

Two explanations might be advanced for the unequal behavior of the various acids in the presence of malonate. The first involves the assumption that malonate acts specifically on succinic dehydrogenase. Since the amounts of fumarate and succinate normally found in muscle are small, their effect would be completely wiped out by added malonate, and respiration would then depend upon the rapidity with which the various acids added could restore these substances to a concentration sufficient to function in hydrogen transport. The ineffectiveness of citrate (and glutamate) in restoring the respiration of malonate-poisoned tissue would therefore suggest a slow or inefficient conversion of citrate to substances necessary in respiration, such as succinate-fumarate. The failure of citrate to restore respiration even when added 30 minutes prior to the malonate only emphasizes this inefficient conversion. However, if citrate is converted to other substances only slowly, or with difficulty, this would exclude it as a member of an essential respiration cycle, since a basic prerequisite for such a cycle is the ready interconversion of its components.

But citrate unquestionably catalyzes respiration in the absence

of malonate (6, 10, 14). This catalysis, however, might not be due to the citrate itself, but rather to catalytic substances formed from citrate in the presence of tissue, such as the  $C_4$  acids of Szent-Györgyi. This idea has been expressed by Elliott and Elliott (15) and Szent-Györgyi (9). It is given some support by the fact that with malonate, glutamate acts much like citrate. Glutamate has not been postulated as a member of the citric acid cycle, but is known to yield  $\alpha$ -ketoglutarate on contact with respiring tissue.

A somewhat different interpretation of our results involves the suggestion of Weil-Malherbe (7) that malonate is not a specific inhibitor of succinic dehydrogenase, but that it also inhibits other systems in intact tissue. Whereas increased amounts of succinate completely compensated for malonate inhibition, extra citrate or glutamate failed to do so, suggesting a greater, or more permanent malonate sensitivity of the citrate and glutamate systems than succinic dehydrogenase itself. Isocitric dehydrogenase, however, is not sensitive to malonate (16).

If non-specific inhibition of malonate be assumed, it is still difficult to reconcile our results with the theory of a citric acid cycle essential for respiration. The inhibition of respiration by malonate in the presence of citrate could be attributed to an interference with the degradation of citrate, an essential reaction in the citric acid cycle. But if this be true, neither fumarate, malate,  $\alpha$ -ketoglutarate, nor succinate should have been able to restore respiration in the presence of malonate, since they can hardly be concerned with citrate degradation. Hence, it is possible that whereas part of the citric acid cycle may be essential, the entire cycle as such is not; and furthermore, that the component parts of the cycle can break down in other ways than those postulated. Thus  $\alpha$ -ketoglutarate is said to be intermediate between citrate and succinate: citrate  $\rightarrow \rightarrow \alpha$ -ketoglutarate  $\rightarrow$  succinate (6, 17, 18). One might therefore expect its respiratory activity to be like that of citrate or succinate, or somewhat between the two. In the presence of malonate, however,  $\alpha$ -ketoglutarate was definitely more effective in restoring respiration than succinate, and very much more effective than citrate. The experiments of Hallman and Simola (19) likewise suggest other pathways of

degradation.  $\alpha$ -Ketoglutarate incubated with muscle yielded much more citrate than did any of the other members of the cycle.

#### SUMMARY

1. The respiration of pigeon breast muscle inhibited by malonate was effectively restored by the addition of fumarate, malate, or  $\alpha$ -ketoglutarate. Succinate also restored the respiration, but relatively more was needed. Citrate and glutamate completely failed to restore respiration when 0.005 M malonate was used, and were inferior to the other acids in the presence of lower amounts of malonate.

2. Glutamic acid, like citric,  $\alpha$ -ketoglutaric, and the  $C_4$  acids, stimulated respiration catalytically.

3. An intact citric acid cycle does not appear to be essential for the respiration of muscle. The same conclusion is reached whether malonate is regarded as a general inhibitor, or as a specific inhibitor for succinic dehydrogenase.

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## DETERMINATION OF PARALDEHYDE IN BIOLOGICAL FLUIDS\*

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In order to investigate quantitatively the concentrations of paraldehyde in blood and urine at various intervals of time after the administration of the drug we were in need of a simple, accurate, and rapid method in which only small quantities of fluid would be required for analysis. Apparently the only published method for the determination of paraldehyde in biological fluids is that of Nitzescu, Georgescu, and Timus (1). This method is based on the depolymerization of paraldehyde by sulfuric acid and steam distillation of the acetaldehyde thus formed into a sodium bisulfite solution. The bisulfite-acetaldehyde complex is decomposed with sodium bicarbonate and the bisulfite determined by titration with standard iodine solution. No recovery data are given by these investigators, nor do they state within what limits of error the method is applicable.

To satisfy our particular requirements we have developed a method based on the oxidation of paraldehyde to acetic acid in the presence of an excess of a potassium dichromate-sulfuric acid mixture. With essentially the same type of apparatus that was employed by Newman (2) for the determination of ethyl alcohol, the paraldehyde is removed from the specimen of fluid by vacuum distillation and bubbled through the oxidizing mixture. In the presence of the high concentration of mineral acid the paraldehyde depolymerizes to acetaldehyde which is immediately oxidized to

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acetic acid. The excess dichromate is determined by iodometric titration with sodium thiosulfate.

*Reagents—*

Potassium dichromate-sulfuric acid oxidizing reagent. To 1 volume of approximately 0.1 N potassium dichromate add 1 volume of concentrated sulfuric acid. The acid should be added slowly and with adequate cooling.

Sodium thiosulfate stock solution. Dissolve 24.82 gm. of sodium thiosulfate in water; add 2 ml. of 10 per cent sodium hydroxide and dilute to 1 liter with distilled water.

Sodium thiosulfate, 0.025 N. This is prepared by diluting the stock thiosulfate and standardizing against potassium iodate. 1 ml. of 0.025 N sodium thiosulfate is equivalent to 0.5504 mg. of paraldehyde.

Potassium iodide solution, 40 per cent.

Starch solution, 1 per cent.

*Procedure*

Cover the bottom of a 50 ml. Pyrex Erlenmeyer flask evenly with a thin layer of about 8 to 10 gm. of anhydrous sodium sulfate (this prevents foaming). Pipette onto this layer 2 ml. (1 ml. may be used if desired) of the fluid to be analyzed and stopper tightly until ready for analysis. The apparatus is set up as shown in Fig. 1. The inlet tube to the oxidizing mixture should have a perforated bulb bottom to reduce the size of the bubbles produced on distillation. For suction an efficient oil vacuum pump must be used. The temperature of the water bath should be kept between 65–70°.

When the sample is ready for analysis, pipette 10 ml. of the dichromate reagent into the oxidizing tube (C). Insert the stopper firmly into the neck of this tube, and tighten screw-clamp *D* of the outlet tube to the vacuum pump. With stop-cock *A* of the inlet tube to the Erlenmeyer flask closed, insert firmly the 2-holed rubber stopper *B* and suspend the flask in the water bath. Start the suction and gradually open the screw-clamp *D*. In the event that foam develops in the Erlenmeyer flask, it may be broken by opening momentarily stop-cock *A*. This operation may be repeated if necessary. We have experienced no trouble from foaming when a sufficient amount of sodium sulfate was

spread evenly on the bottom of the Erlenmeyer flask. The vacuum is maintained for 15 minutes. To break the vacuum, screw-clamp *D*, leading to the pump, is closed and stop-cock *A* of the inlet tube to the Erlenmeyer flask is gradually opened.

The dichromate reagent in the oxidizing tube and that adhering to the inside and outside walls of the inlet tube are washed into a 300 ml. Erlenmeyer flask and diluted to about 100 ml. with distilled water. Add 10 ml. of the 40 per cent potassium iodide

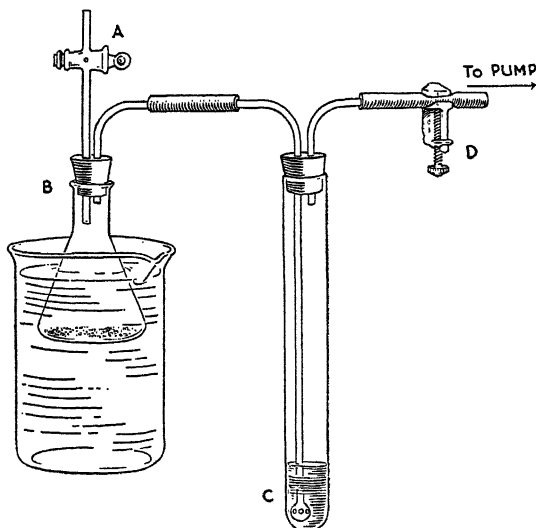


FIG. 1. Apparatus for the determination of paraldehyde in biological fluids. *A* stop-cock of inlet tube, *B* 2-holed rubber stopper, *C* oxidizing tube, *D* screw-clamp.

solution and titrate with 0.025 *N* sodium thiosulfate, with starch as indicator. It is preferable to add the starch just before the end-point, when the color of the solution has changed from yellow to a yellowish green. Since blood and urine contain small amounts of volatile oxidizable substances (3, 4), blank determinations are run with similar amounts of the corresponding biological fluid which is known to contain no added paraldehyde, alcohol, or other volatile oxidizable substances. For clinical purposes the small quantities of oxidizable substance present may be disregarded and the blank determination made by pipetting 10 ml. of the dichro-

mate reagent into a 300 ml. Erlenmeyer flask, diluting to about 100 ml. with distilled water, adding 10 ml. of the potassium iodide solution, and titrating with the thiosulfate.

The chemical reactions are represented by the following equations.

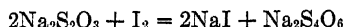
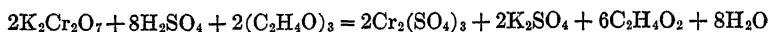


TABLE I

*Recovery of Known Amounts of Paraldehyde Added to Biological Fluids*  
In each case 2 ml. of fluid were taken for analysis.

Fluid	Paraldehyde added to 100 ml. fluid	Paraldehyde recovered	Per cent recovery
	<i>mg.</i>	<i>mg.</i>	
Water	25	24	96.0
	64	63	98.4
	70	71	101.4
	105	105	100.0
	135	131	97.0
	147	145	98.6
	235	238	101.3
Urine	60	60	100.0
	115	114	99.1
	145	145	100.0
	149	149	100.0
	239	242	101.3
Blood	18	17	94.4
	23	22	96.0
	51	50	98.0
	55	54	98.0
	62	60	97.0
	134	132	99.0
	148	145	98.0
	205	204	99.5

### Calculation

$((B - A) \times 0.5504/V) \times 100 = \text{mg. per 100 ml. of fluid,}$   
where  $B$  = ml. of thiosulfate required to titrate the blank,  
 $A$  = ml. of thiosulfate required to titrate the unknown, and  
 $V$  = ml. of sample taken for analysis.

The volume of reagent used will permit determination of paraldehyde concentrations up to 500 mg. per cent with 2 ml. samples and 1 gm. per cent with 1 ml. samples.

The accuracy obtainable by this method is illustrated by the data in Table I.

#### DISCUSSION

The values given in Table I are approximately of the order of magnitude likely to be encountered in blood and urine following the use of paraldehyde in clinical and experimental studies. When a microburette graduated in 0.01 ml. was used, duplicate determinations were found to check within 1.5 mg. per cent. With an ordinary burette graduated in 0.1 ml., duplicate determinations usually checked within 3 mg. per cent.

Since this method is based on the removal of paraldehyde from the body fluid by vacuum distillation, it is impossible by this technique to separate it from other volatile oxidizable substances. For this reason the method is not applicable in the presence of abnormal concentrations of such volatile substances as alcohol or acetone. By this method normal human blood was found to contain from 6 to 10 mg. per cent of volatile oxidizable material, expressed as paraldehyde. Normal urine was found to contain up to 4 mg. per cent. Dog blood contained about 3 mg. per cent. Since the accuracy of the method is probably no greater than within 3 mg. per cent, the values for normal dog blood and human urine fall nearly within the range of error of the analytical procedure. However, it is of interest to note that if the concentrations of the volatile reducing substances in human and dog blood were expressed as ethyl alcohol, the value obtained would be about 5 mg. per cent for human blood and approximately 1.4 mg. per cent for dog blood. Gettler, Niederl, and Benedetti-Pichler (4), using an especially refined technique, reported the average amount of alcohol contained in normal human blood to be approximately 4 mg. per cent and in dog blood about 1.3 mg. per cent. As there are minute concentrations of volatile oxidizable materials other than alcohol, the results obtained in our control analyses of human and dog blood are in very close agreement with those of Gettler and his associates.

## SUMMARY

A simple, rapid, and accurate method is described for the determination of paraldehyde in biological fluids. The paraldehyde is removed from the biological fluid by vacuum distillation and bubbled through a potassium dichromate-sulfuric acid oxidizing mixture. The excess dichromate is titrated iodometrically with sodium thiosulfate.

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# THE EXCRETION OF VOLATILE SELENIUM COMPOUNDS AFTER THE ADMINISTRATION OF SODIUM SELENITE TO WHITE RATS\*

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It is commonly stated (1) that after the administration of selenium or tellurium salts, volatile methyl compounds, dimethyl selenide or telluride, are excreted through the lungs. This methylation has long been regarded as one of the classical examples of detoxication by methylation and as such is important biologically. When the exhaled gases of a dog injected with sodium tellurite were passed through a solution of iodine in potassium iodide and the resultant solution was treated with alkali and sodium sulfide, the odor of dimethyl sulfide was evolved. Treatment with reducing agents resulted in the precipitation of metallic tellurium. A characteristic garlic-like odor resembling that of dimethyl telluride was observed in the breath of the experimental animals. On the basis of this evidence, Hofmeister (2) concluded that the excretory product was dimethyl telluride.

The greater toxicity of selenium salts prevented the administration of sufficiently large amounts of sodium selenite to make possible the repetition of the above experiments. Since the odor in the breath of animals injected with selenium salts was similar to that of synthetic dimethyl selenide and since selenium and tellurium are so closely related chemically, Hofmeister was led to believe that selenium also was detoxicated and excreted as the dimethyl derivative (2). Maasen (3) repeated Hofmeister's experiments but did not extend his findings. Although as pointed

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out by Challenger (4), "this conclusion lacks experimental verification," acceptance of the biological methylation of tellurium and selenium in the animal organism has been general. The methylation of these elements by microbial agencies has, however, been definitely established (4).

The excretion of selenium through the kidneys and gut has been studied extensively (5, 6). We have been unable to find recorded any quantitative study of the excretion of volatile compounds of selenium in animals injected with selenium salts. The present investigation is concerned with this problem. Since it is usually accepted that the chief volatile product is dimethyl selenide, substances which might be expected to furnish methyl groups in metabolism (*e.g.*, choline and methionine) and to facilitate thus the excretion of selenium in volatile form were administered in some experiments to the animals which received injections of sodium selenite.

#### EXPERIMENTAL

Adult male white rats were injected subcutaneously with a solution of sodium selenite containing approximately 1 mg. of selenium per cc. at a level of 2.5 to 3.5 mg. per kilo of body weight. The rats were then placed in a respiration apparatus and the exhaled air was analyzed as described subsequently.

In some experiments the animals received choline chloride or methionine in addition to the selenium salt. Methionine was fed as the sodium salt through a stomach tube. Single doses of choline chloride were injected in aqueous solution with the sodium selenite. In other experiments with repeated administration of choline, a somewhat different procedure was followed. On the preliminary days and on the day of the selenium injection, the rats received a small piece of bread which had been moistened with the desired amount of choline chloride solution. This was readily consumed and the animals were then fed the standard laboratory stock diet of whole wheat bread, milk, and lettuce. Control animals received the bread without the addition of the choline salt. In each case in which a supplement was administered with the selenite, a control animal which received no supplement was injected with the selenite. The pairs of animals thus studied are designated in Table II by the same letters. Two

animals (designated in Table II by the same letter and injection number) were always studied in parallel experiments on the same day.

The choice of an absorbent for any volatile selenium compounds presented some difficulty. A number of absorbents for dimethyl selenide have been described and used in the study of the bacterial metabolism of selenium compounds (4). Nitric acid was found to remove the volatile selenium compounds from the expired air of our experimental animals effectively. Its use in the quantitative studies was, however, not entirely satisfactory. Since it has been generally accepted that the volatile selenium compound is dimethyl selenide and since alkyl selenides like ethers or thio ethers (7) should be soluble in concentrated sulfuric acid, experiments were carried out to see whether this absorbent would remove volatile selenium compounds from the respiratory gases. Since it was proposed to determine the selenium colorimetrically by the use of the reaction of selenium salts with codeine (8, 9) and since this reaction takes place in concentrated sulfuric acid, the desirability of the use of this absorbent was obvious.

After the injections the animals were placed in the usual type of round wire cage inside a respiration chamber (large carboy from which the bottom had been removed) which permitted collection of the respired gases and through which a current of air was slowly drawn. The gases were passed successively through two tubes containing hydrochloric acid (1:1), a tube containing 20 per cent sodium hydroxide solution, and a large absorption tube containing glass wool and solid granulated calcium chloride. The dry gases then entered a specially designed all-glass absorption tube, constructed according to the principle suggested by Nichols (10) but of smaller size, which insured intimate contact of the gases with the absorbent. In this tube approximately 45 cc. of concentrated sulfuric acid were placed. The gases then passed through a second tube containing sulfuric acid which was connected with a vacuum water pump.

The two absorption tubes containing sulfuric acid are shown in Fig. 1. The efficiency of this type of absorption chamber is discussed by Nichols (10). The tubes shown were filled with acid by closing the stop-cock at the inlet, opening the stop-cock at the bottom of the tube, and attaching a vacuum water pump to the

point of exit of the system. We have also used an apparatus in which Tubes A and B were connected by a ground joint and the absorption apparatus was connected with the chamber containing the acid by ground joints.

When the current of air was properly regulated, all the selenium was found in the first absorption tube containing sulfuric acid and no selenium was present in the second sulfuric acid tube. Accordingly, our analyses were confined to the contents of the

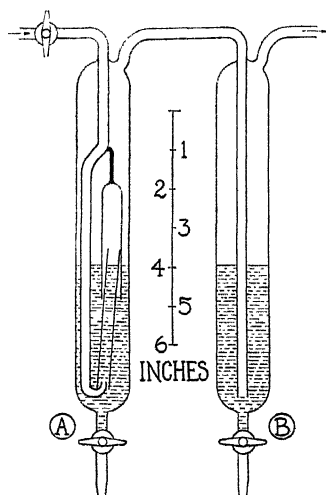


FIG. 1. Tubes A and B which served as containers for the sulfuric acid in the absorption of volatile selenium compounds.

first of the two tubes containing sulfuric acid. The acid was transferred from the absorption tube to a Kjeldahl flask, 1 cc. of 30 per cent hydrogen peroxide was added, and the mixture was gently heated for from 20 to 30 minutes on a Kjeldahl digestion rack. After cooling, the volume of the digest was measured and 10 cc. portions were transferred to test-tubes. 2 drops of an aqueous 4 per cent solution of codeine phosphate were added, the solution being cooled under the tap. The tubes were stoppered and placed in the dark for 20 to 30 minutes.<sup>1</sup> The colors were compared in a

<sup>1</sup> Gortner and Lewis (9) observed that with digests of tissues and feces which contained selenium, 5 to 6 hours were necessary for the maximal

colorimeter with that developed by 10 cc. of a standard solution of sodium selenite in concentrated sulfuric acid. To this standard, codeine phosphate was added and 20 to 30 minutes were allowed for the development of the color, as with the unknown. In early experiments, a series of standards containing from 0.03 to 0.1 mg.

TABLE I

*Determination of Selenium in Solutions of Known Concentrations of Sodium Selenite and in Digests from Absorbed Products of Respiratory Gases*

Unless otherwise indicated, the standard solution used contained 0.1 mg. of selenium. The standards were all set at 20 mm.

	Selenium present	Colorimetric reading	Selenium found
	<i>mg.</i>	<i>mm.</i>	<i>mg.</i>
Series I	0.1	20.4	0.098
	0.08	26.0	0.077
	0.06	33.0	0.061
	0.06	30.0	0.067
" II	0.1	17.3	0.115
	0.08	23.7	0.084
	0.06	29.5	0.068
" III	0.05	20.0*	0.05
	0.08	11.9*	0.084
Unknown Solution 411		19.1†	0.084
		24.0	0.083
" " 4212		26.8	0.0745
		13.5*	0.074
" " 4213		31.7	0.063
		17.5*	0.0575

\* Read against a standard containing 0.05 mg. of selenium.

† Read against a standard containing 0.08 mg. of selenium.

of selenium per 10 cc. was prepared and the standard whose reaction color most nearly resembled that of the unknown was used for the more exact comparison in the colorimeter. It was found, however, that the accuracy obtained with standards of 0.05 and 0.1 mg. was sufficient to permit determination of the individual

development of color in the codeine-selenium reaction. The presence of inorganic salts in the digest may have influenced the rate of color development. The digests of the present series contained relatively little material other than the selenium derivatives under discussion.

variations of the excretion of volatile selenium compounds. This is shown in Table I, in which are presented typical recoveries of varying amounts of selenium within our experimental range when

TABLE II

*Excretion of Volatile Selenium Compounds after Subcutaneous Injection of Sodium Selenite*

The letters following the rat numbers refer to the pairs of rats. In each case, the pair received the selenium injection on the same day and, when choline or methionine was fed to one animal, its control received no supplement. Except where indicated, the experimental period extended over 8 hours.

Rat No.	Injection No.	Weight	Selenium				
			Injected		Excreted in gases		
				Per kilo body weight		Per cent of amount injected	Per kilo body weight
		mg.	mg.	mg.	mg.		mg.
4161-A	1	372	1.80	3.5			
	2	350	1.05	3.0	0.298	28	0.9
	3*	320	1.00	3.0	0.459	46	1.4
	4†	310	0.90	2.9	0.264	28	0.8
4181-A	1	397	1.40	3.5	0.687‡	49	1.7
	2	335	1.00	3.0	0.303	30	0.9
	3	328	1.00	3.0	0.340	34	1.1
	4	314	0.90	2.9	0.164	18	0.4
4201-B	1	350	1.20	3.4	0.625	52	1.8
	2§	316	0.95	3.0	0.153	16	0.5
	3*	307	0.90	2.9	0.303	34	1.0
	4†	277	0.83	3.0	0.230	28	0.8
4202-B	1§	340	1.20	3.5	0.650	54	1.9
	2	286	0.85	3.0	0.145	17	0.5
	3	272	0.71	2.6	0.242	34	0.9
	4	256	0.77	3.0	0.222	29	0.9
4212-C	1	253	0.75	3.0	0.335	45	1.3
	2§	247	0.67	2.7	0.152‡	23	0.6
	3	200	0.60	3.0	0.178	30	0.9
	4*	232	0.70	3.0	0.228	33	1.0
4213-C	5†	213	0.65	3.0	0.470	72	2.1
	1§	238	0.70	3.1	0.295	42	1.2
	2	212	0.65	3.1	0.195‡	30	0.9
	3	200	0.60	3.0	0.169	28	0.8
	4	217	0.70	3.2	0.198	28	0.9
	5	211	0.65	3.1	0.162	25	0.8

TABLE II—*Concluded*

Rat No.	Injection No.	Weight	Selenium				
			Injected		Excreted in gases		
				Per kilo body weight		Per cent of amount injected	Per kilo body weight
		mg.	mg.	mg.	mg.		mg.
4221-D	1	208	0.60	2.9	0.202	34	1.0
	2	206	0.60	2.9	0.248	41	1.2
	3*	203	0.60	3.0	0.210	35	1.0
	4†	196	0.60	3.1	0.210	35	1.1
4231-D	1§	223	0.65	2.9	0.207	32	0.9
	2	232	0.69	3.0	0.328	47	1.4
	3	227	0.70	3.1	0.250	35	1.1
	4	221	0.65	2.9	0.228	35	1.1

\* Injection of 50 mg. of choline chloride at the same time as the selenium injection. Rat 4221-D received only 30 mg.

† The animals received 100 mg. of choline chloride daily in the diet for 3 to 5 days previous to the selenium injection.

‡ Experimental period 24 hours.

§ Received 0.454 gm. of methionine 3 to 4 hours before the injection of selenium.

|| Experimental period 9 hours.

read against 0.05 or 0.1 mg. standards. The results presented for three typical unknowns show essentially the same values with standards of 0.05 to 0.1 mg. We were convinced from this and similar control experiments that the method was sufficiently accurate for our purposes.

The results of our experiments are presented in Table II. Rats which received 2.5 to 3.5 mg. per kilo of selenium as sodium selenite without either choline or methionine excreted within 8 hours from 17 to 52 per cent of the selenium injected as a volatile selenium compound which could be absorbed by concentrated sulfuric acid. This corresponded to an excretion of 0.5 to 1.8 mg. of selenium per kilo. The administration of either methionine or choline chloride failed to influence the excretion of the volatile selenium compounds with two possible exceptions. Rat 4202-B excreted 54 per cent of the selenium injected in volatile form after the first injection when methionine was fed. The amount excreted after the subsequent injections was significantly less, but

it should also be noted that the paired mate of the animal, Rat 4201-B, excreted 52 per cent even though no methionine were fed. Rat 4212-C which received daily feedings of 100 mg. of choline chloride for several days previous to the fifth injection of sodium selenite excreted 72 per cent, or 2.1 mg. of selenium per kilo. This is the highest value observed in thirty-three determinations.

In twenty experiments in which selenite was injected, the average excretion of selenium as volatile selenium compounds absorbed by concentrated sulfuric acid was 1.0 mg. per kilo of body weight. When choline chloride or methionine was administered also, the average excretions per kilo of body weight were 1.0 mg. of selenium in seven experiments with choline and 0.8 mg. in four experiments with methionine. The two extraordinarily high values discussed have been omitted in the calculation of these average values. It is evident that the excretion of volatile selenium compounds does not vary significantly and under our experimental conditions is not materially influenced by the ingestion of choline or methionine. This is of interest in view of the recent studies of methionine and choline in relation to fatty livers (11) and methylation of homocystine (12).

The values represent, so far as we have been able to discover, the first quantitative study of this kind. The excretion of selenium in the urine, feces, and bile has been determined. Gortner and Lewis (6) found that 18 to 70 per cent of *orally* ingested selenium in experiments extending over considerable periods of time was excreted in the feces, although over 50 per cent of the rats used excreted from 21 to 39 per cent of the ingested selenium in the feces. Smith and coworkers (5) found that the cat excreted from 50 to 80 per cent of *injected* selenium in the urine.

The rat, in contrast to the cat, is not readily susceptible to intoxication with sodium selenite. This lower toxicity in the rat may be related to the rapid formation and excretion of volatile selenium products through the lungs, while in the more susceptible cat, the selenium may combine with the tissues and be excreted more slowly. In the rat, 30 to 40 per cent of the injected selenium was found to be excreted in volatile form in 8 hours, while in the cat, 24 to 50 hours were required to excrete the same percentage of the ingested selenium in the urine.

In conclusion, it may be pointed out that the present study does not afford evidence of the nature of the volatile selenium com-

pound, other than its property of being absorbed by concentrated sulfuric acid. Further studies designed to demonstrate whether this substance is actually dimethyl selenide, as postulated by Hofmeister (2), are in progress. The possibility that other volatile selenium compounds, not absorbed by concentrated sulfuric acid, are also present has not, of course, been excluded.

#### SUMMARY

When sodium selenite (2.5 to 3.5 mg. of selenium per kilo) was injected subcutaneously into adult white rats, from 17 to 52 per cent of the injected selenium was excreted within 8 hours as a volatile compound which was absorbed by concentrated sulfuric acid.

Under the conditions of these experiments, the excretion of this type of volatile selenium compound was not increased by the administration of either methionine or choline chloride. Since Hofmeister had postulated that the volatile selenium compound excreted was dimethyl selenide, these compounds were administered as potential sources of methyl groups which might assure more ready methylation. It is pointed out, however, that Hofmeister's hypothesis of the nature of the selenium compound lacks adequate experimental proof.

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## THE DISTRIBUTION OF KETONE BODIES IN TISSUES\*

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Evidence has been accumulating that the liver is the chief site of ketogenesis and that destruction of ketone bodies occurs mainly in the extrahepatic tissues. Embden and Kalberlah (1) and subsequently Snapper, Grünbaum, and Neuberg (2), using the perfusion technique of Embden, offered experimental evidence that the liver is the site of ketone body formation. The results of *in vitro* studies of Quastel and Wheatley (3), Jowett and Quastel (4), and Edson (5) have all supported this hypothesis. Mirsky (6) showed that completely eviscerated rabbits fail to develop a ketosis in response to injection of anterior pituitary extract, while rabbits in which only the liver remains intact do respond to the ketogenic action of these extracts. Evidence that destruction of ketone bodies is mainly the function of the extrahepatic tissues has been furnished by the same authors (2, 7-11).

The present experiments were undertaken in an attempt to study simultaneously in the intact animal the production and the utilization of ketone bodies, by determination of the concentrations of these substances in liver and muscle cells at various stages during the ketosis produced by several methods.

### *Chemical Methods*

Total ketone bodies (acetone, acetoacetic, and  $\beta$ -hydroxybutyric acids) of blood and tissues were determined by a slight modification of the Van Slyke and Fitz method (12), the difference being in the method of precipitation of proteins and other interfering substances.

\* The data in this paper are taken in part from a dissertation presented by Helen C. Harrison in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1939.

Samples of liver or muscle are frozen immediately upon removal from the animal and kept in a frozen state until analysis is possible. The tissue is weighed in the frozen state. A sample of liver or muscle weighing approximately 4 gm. is cut into small pieces, ground thoroughly in a mortar with clean sand and a small amount of water, and the macerated tissue transferred quantitatively to a 250 cc. volumetric flask, about 50 cc. of distilled water being used for this operation. The protein and other interfering substances are precipitated with 50 cc. of a 20 per cent copper sulfate solution and an equal volume of 10 per cent calcium

TABLE I

*Recovery of Added  $\beta$ -Hydroxybutyric Acid from Muscle and Liver*

$\beta$ -Hydroxybutyric acid added	Muscle		Liver	
	Acid recovered	Per cent recovered	Acid recovered	Per cent recovered
<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	
1.11	0.96	86.0	1.08	97.5
1.11			1.21	109.0
1.20	1.23	102.5		
1.20	1.21	100.7		
1.20	1.21	100.7		
2.22	2.27	102.0	2.15	97.0
2.22	2.15	97.0	2.26	102.0
2.22	2.29	103.0	2.29	103.0
5.56	6.08	109.0	5.78	104.0
5.56	6.24	112.0	5.81	104.5
11.12	13.1	118.0	11.85	106.5

hydroxide suspension. A similar technique of protein precipitation has been used by Edson (5). The determination of total acetone bodies in the filtrate is carried out by the gravimetric method of Van Slyke and Fitz (12), with small sintered glass filters, No. 10G4.

The reliability of the method was checked by recovery experiments. Weighed amounts of *dl*- $\beta$ -hydroxybutyric acid were added to samples of liver and muscle of approximately 4 gm. in weight and determinations of  $\beta$ -hydroxybutyric acid made. The results are given in Table I. In the analyses of tissues reported in the present investigation, the concentrations of

$\beta$ -hydroxybutyric acid determined were in the range in which the recoveries were equal to the theoretical amounts present. In the interpretation of results, the accuracy of the method is taken to be  $\pm 10$  per cent.

The copper sulfate-calcium hydroxide method of protein precipitation was used in preparing blood filtrates and total ketones were determined in the same manner as described for tissues.

### *Tissue Analysis*

From the determination of the concentration of ketone bodies in plasma, liver, and muscle, estimations of the concentrations of these substances within the liver and muscle cells were made according to the method of Harrison, Darrow, and Yannet (13). The proportions of extracellular and intracellular water of rat liver and muscle have been determined by Harrison and Darrow (14), and their results have been used. The concentration of ketones in the extracellular water is assumed to be the same as in plasma water. The actual method of calculation may best be demonstrated by an example.

If Ket. is used to represent the concentration of total ketones, the concentration in muscle cell water may be calculated as shown in the following specific example.

$$\begin{aligned}\text{Ket.}_{\text{plasma}} &= 76.7 \text{ mg. per 100 cc. (by analysis)} \\ \text{Ket.}_{\text{plasma water}} &\text{ is then } 76.7/0.93 = 82.5 \text{ mg. per 100 cc.} \\ \text{Ket.}_{\text{muscle}} &= 11.3 \text{ mg. per 100 gm. (by analysis)}\end{aligned}$$

Since 12 per cent of muscle weight is extracellular water, in which the concentration of ketones is the same as in plasma water, the amount of ketones present in the extracellular fluids of muscle is calculated to be  $\text{Ket.}_{\text{plasma water}} \times 0.12 = 9.9 \text{ mg.}$

If this amount be subtracted from the total ketone body content of muscle, the amount of ketones present in the muscle cells is obtained. The intracellular water of muscle is taken to be 63 per cent of the fresh muscle weight. Therefore, the concentration of ketones in muscle cell water is  $1.4/0.63 = 2.2 \text{ mg. per 100 cc. of cell water.}$

Similar calculations can be made for liver, in which the extracellular water is taken to be 20 per cent and the intracellular water 50 per cent of the weight of the liver tissue.

*Procedure*

Male rats from a single colony, weighing between 150 and 200 gm., were used. Five groups of experiments were performed. In Group I rats were fasted after receiving the stock diet of Purina Fox Chow and the animals sacrificed after 24 to 72 hours of fasting. Group II was fasted for 48 hours and then injected subcutaneously with from 0.8 to 1.0 cc. of an alkaline extract of beef anterior pituitary, prepared according to the method of Burn and Ling (15). The animals were sacrificed 2 hours after the injection. Ketosis was induced in Group III by fasting rats for 72 hours after a high fat diet (40 per cent fat) was fed. Group IV was fasted and 50 mg. of phlorhizin in 1 cc. of olive oil were injected subcutaneously. Of this group, some rats were fasted 24 hours, injected with phlorhizin, and sacrificed 6 hours after the injection. Others were fasted 48 hours and injected once daily with phlorhizin during this time. The ketosis studied in these four groups may be considered to be of endogenous origin.

In Group V ketosis was induced by the administration of  $\beta$ -hydroxybutyric acid. The *dl*- $\beta$ -hydroxybutyric acid, neutralized to pH 7.4 with N sodium hydroxide, was administered either by intraperitoneal injection or by gavage to rats fasted 24 hours. Varying amounts of the salt were given and the animals sacrificed after 4 hours.

At the end of the experimental period all animals were anesthetized with nembutal. Blood was taken from the abdominal aorta, the entire liver removed, and a sample of muscle taken from the hind extremities for analysis. The liver and muscle were frozen immediately upon removal. Total ketone bodies were determined by the method described and calculation of the concentration within the liver and muscle cells made. The results are given in Tables II and III.

*Results*

In Table II the results obtained in the experiments when the ketosis was of endogenous origin are given. The ratio of the concentrations of ketone bodies in liver cell water to plasma water,  $\text{Ket.}_l/\text{Ket.}_p$ , and in muscle cell water to plasma water,  $\text{Ket.}_m/\text{Ket.}_p$ , are shown in the last two columns of Table II. The data are arranged according to increasing concentrations of

TABLE II

*Distribution of Ketone Bodies\* in Blood, Liver, and Muscle Following Production of Ketosis by Fasting, Anterior Pituitary Extract, and Phlorhizin*

Group No.	Blood	Liver	Muscle	Plasma H <sub>2</sub> O	Liver cell H <sub>2</sub> O	Muscle cell H <sub>2</sub> O	Ket. <sub>l</sub> Ket. <sub>p</sub>	Ket. <sub>m</sub> Ket. <sub>p</sub>
	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		
I. Fasted 48 hrs.	1.4	4.0		2.0				
after stock	1.4		0	2.0				
diet	2.6	5.2	0	3.6				
	4.0	4.2		5.6				
	4.5	4.9		7.0				
	12.5†	11.2	0	17.5	15.4	0	0.89	0
	21.7†	17.2	0	30.4	22.2	0	0.73	0
	28.0	21.0	3.1	39.2	26.2	0	0.67	0
	36.5†	24.7	8.0	51.0	29.0	3.0	0.57	0.06
II. Fasted; injected	7.8	6.6		10.9	8.8		0.81	
with anterior	16.7	15.5		23.3	21.6		0.93	
pituitary ex-	17.2	10.1		24.0	10.6		0.44	
tract	19.1		0.9	26.6		0		0
	22.0	23.1	3.0	30.8	33.8	0	1.09	0
	28.8	17.7	2.1	40.4	19.5	0	0.44	0
	30.0		3.3	42.0		0		0
	34.4	37.7		47.0	56.2		1.17	
	39.5	30.3		55.1	38.6		0.70	
	39.6	22.0	6.9	55.4	21.8	0	0.39	0
	59.0	53.5	11.3	82.5	74.0	2.2	0.90	0.03
III. Fasted 72 hrs.	55.5	38.8	10.6	77.5	46.6	2.1	0.60	0.03
after high fat	58.3	30.4	21.4	81.5	28.2	18.4	0.35	0.23
diet	65.4	48.1	18.9	91.3	59.8	12.6	0.65	0.14
	66.6	37.5	20.3	93.0	37.8	14.5	0.41	0.16
IV. Fasted; injected	27.4	17.0		38.2	18.6		0.49	
with phlorhi-	45.5	36.1	14.7	63.5	46.8	11.1	0.74	0.17
zin	82.0	61.5	27.1	114.5	67.0	21.1	0.59	0.18
	89.6	69.0	22.9	125.0	88.0	12.5	0.70	0.10
	120.4	86.6	50.4	168.0	106.0	48.0	0.63	0.29
	134.0	124.5	54.0	187.0	174.2	50.0	0.93	0.27
	140.0	88.5	61.2	195.5	99.0	60.0	0.51	0.31
	163.5	130.0	90.5	228.0	169.0	100.0	0.74	0.44
	179.5	123.8	72.0	251.0	147.2	66.0	0.59	0.26
	268.0	314.0	139.0	375.0	478.0	149.0	1.28	0.40
Average.							0.70	

\* Expressed as  $\beta$ -hydroxybutyric acid.

† Female rats, fasted 72 hours.

ketone bodies in plasma within each of the four groups. It is apparent from Table II that the findings in liver and muscle are markedly different.

TABLE III

*Distribution in Liver and Muscle of  $\beta$ -Hydroxybutyric Acid 4 Hours after Injection*

Rats fasted 24 hours;  $\beta$ -hydroxybutyric acid injected intraperitoneally or by stomach tube in varying amounts; rats sacrificed 4 hours after administration of acid.

Whole blood	Liver	Muscle	Plasma H <sub>2</sub> O	Liver cell H <sub>2</sub> O	Muscle cell H <sub>2</sub> O	$\frac{\text{Ket.}_l}{\text{Ket.}_p}$	$\frac{\text{Ket.}_m}{\text{Ket.}_p}$
<i>mg. per 100 cc.</i>	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		
8.4	5.2	0	11.8	5.6	0	0.47	0
12.5	12.7	1.2	17.5	18.4	0	1.05	0
18.7	8.7	0	26.5	6.8	0	0.26	0
22.0	22.7	7.2	30.4	33.2	5.6	1.10	0.18
23.1	10.5	2.7	32.3	8.0	0	0.25	0
36.8	14.1	3.0	39.6	12.4	0	0.31	0
27.3	13.3	0	38.1	11.4	0	0.30	0
27.4	20.6	2.6	35.6	27.0	0	0.76	0
27.9	31.0	5.8	39.0	46.4	0	1.18	0
32.5	19.1	1.5	34.9	25.4	0	0.73	0
34.8	19.5	3.7	48.8	19.4	0	0.40	0
36.8	20.1	2.5	39.6	24.4	0	0.62	0
38.0	19.3	5.5	53.3	17.2	0	0.32	0
41.8	37.6	0	58.5	51.8	0	0.89	0
48.5	29.0	14.0	68.0	30.8	6.7	0.45	0.10
54.2	45.3	13.0	75.8	60.4	3.9	0.80	0.06
58.4	44.9	13.8	81.8	57.0	6.3	0.70	0.08
61.7	37.5	15.0	86.5	40.4	7.3	0.47	0.08
69.0	42.3	14.7	96.5	46.0	4.9	0.48	0.06
86.8	61.8	21.4	121.5	75.2	7.6	0.62	0.10
121.8	93.2	52.7	170.5	118.4	51.3	0.69	0.30
126.5	83.0	51.9	177.0	95.2	48.8	0.54	0.28
135.2	86.3	45.7	189.0	96.8	36.3	0.51	0.19
154.5	121.0	53.6	216.0	155.2	44.0	0.72	0.20
175.0	97.6	63.6	245.0	97.2	54.2	0.40	0.22
200.0	147.2	83.1	280.0	182.4	78.6	0.65	0.28
Average.....						0.60	

Ketone bodies are found within the liver cell whenever they are found in the plasma. The ratio  $\text{Ket.}_l/\text{Ket.}_p$  is relatively

constant at all levels of plasma concentration. No ratios were calculated for the first few animals in Group I in which the plasma concentration is very low, since at these low levels errors in analyses would invalidate such calculations. It may be seen that there is no systematic difference in the ratio  $\text{Ket.}_1/\text{Ket.}_p$  with increasing concentrations of ketones within a single group or among the different groups.

In the muscle, however, the findings are radically different. At relatively low concentrations of ketones in the plasma no ketones are found in the muscle cell. The small amounts found on analysis of muscle tissue can be approximately accounted for by the estimated concentration in the extracellular water of the muscle. Thus, for example, at plasma concentrations of  $\beta$ -hydroxybutyric acid as high as 50 mg. per 100 cc., no acetone bodies are found in the muscle cell water. At concentrations in the plasma of 70 mg. per 100 cc. and higher, ketones appear in the muscle cell water and the amount increases with increasing concentrations.

The results of the experiments in which *dl*- $\beta$ -hydroxybutyric acid was administered are given in Table III. Here, as in the preceding experiments, the marked difference between liver and muscle is also apparent in the relative constancy of the ratio  $\text{Ket.}_1/\text{Ket.}_p$  and the increasing value of  $\text{Ket.}_m/\text{Ket.}_p$  with increasing plasma concentrations.

#### DISCUSSION

The presence of ketones in the liver cell at the early stages of ketosis is in agreement with the findings of previous investigators that these substances are the product of liver metabolism. The rapidity of response of liver metabolism to treatment with ketogenic substances is shown by the experiments in which phlorhizin was injected as well as by the experiments in which anterior pituitary extract was given. Within 6 hours after the injection of phlorhizin to a rat, previously fasted 24 hours, high levels of ketones are found in the liver and in the plasma. In experiments in which anterior pituitary extract was injected, the animals were sacrificed 2 hours after the injection and at this time there was an increase in concentration of ketones in the liver cell.

The relative constancy of the ratio  $\text{Ket.}_1/\text{Ket.}_p$  at low and high



levels of ketones in the blood plasma indicates that these substances readily diffuse through the liver cell membrane. Accumulation of acetone bodies in unusually high concentration within the liver cell, therefore, does not occur, despite active formation within the liver, but there is an apparent equilibrium between the liver cells and the extracellular fluids. This is, in reality, not a static equilibrium, but rather a "steady state," inasmuch as continued production of ketones within the liver cell is taking place. The apparent paradox of a lower concentration within the liver cell than in the extracellular fluids, despite the fact that the diffusion is from the liver cell to the extracellular fluids, is probably to be explained by the high concentration of protein in the cellular fluid of the liver. Inasmuch as  $\beta$ -hydroxybutyric acid at the pH of the body fluids must exist as an anion, the equilibrium between the cell and the extracellular fluids might be expected to show the relationships predicted by the Donnan theory of membrane equilibrium. Studies of the distribution of  $\beta$ -hydroxybutyric acid and total ketone bodies between red blood cells and plasma were made. The average ratio of the concentration in red blood cell water to that in plasma water was found to be 0.5. This is of the same order of magnitude as the distribution ratio for chlorides as determined by Van Slyke *et al.* (16).

Ketone bodies are not found within the muscle cell at times when they are present in the liver cell and in the extracellular fluids. It is unlikely that the absence of ketone bodies within the muscle cell at such times is due to a relative impermeability of the muscle cell membrane, since perfusion experiments have shown a rapid disappearance of both acetoacetic and  $\beta$ -hydroxybutyric acids from the perfusion fluid upon circulation through muscle. Moreover, the cell membranes of other tissues such as liver and red blood cells are freely permeable to ketone bodies.

If, therefore, the muscle cell membrane is permeable to ketone bodies, the absence of these substances within the muscle cell must be an indication of a rapid transformation of these substances to compounds which no longer react with Denigès's reagent. The disappearance of ketones from the muscle cell must be sufficiently rapid so that at relatively low levels of ketones in the extracellular fluids transformation or destruction of these substances occurs as rapidly as diffusion into the cell, thus permitting no accumulation.

As the concentration outside the cell rises, the rate of diffusion into the cell presumably increases, so that a greater concentration of ketone bodies within the muscle is found. It is not necessary to assume that the increase of ketones in the muscle is the result of impairment of utilization by the muscle cell.

The effect of fasting, anterior pituitary extract, and phlorhizin in producing ketosis is evidently on liver ketogenesis. The similarity in findings in the experiments in which ketosis was of endogenous origin and those in which  $\beta$ -hydroxybutyric acid was injected suggests that the utilization of ketone bodies by muscle tissue is not inhibited following fasting or the administration of anterior pituitary extract and phlorhizin.

#### SUMMARY

1. Determinations of the distribution of ketone bodies between extracellular water and the cell water of rat liver and muscle have shown that these two tissues are entirely different with respect to their metabolism of  $\beta$ -hydroxybutyric and acetoacetic acids.

2. When ketosis of endogenous origin is produced by fasting, treatment with anterior pituitary extract, or injection of phlorhizin, there is found to be at all times an increased concentration of ketone bodies in the liver cell water. The ratio  $\text{Ket.}_l/\text{Ket.}_p$  remains constant, within the limits of error of the techniques employed, at all levels of plasma concentrations.

3. In contrast to the findings in liver, appreciable amounts of ketones are not found in muscle cell water until the concentration in the plasma is elevated to about 70 to 80 mg. per 100 cc. (expressed as  $\beta$ -hydroxybutyric acid). Above this level, as the concentration in the plasma increases, the amount in the muscle cell also increases. The distribution ratio  $\text{Ket.}_m/\text{Ket.}_p$  increases with increasing concentrations of ketones in the plasma, but this value never reaches the value for  $\text{Ket.}_l/\text{Ket.}_p$ .

4. The distribution of ketone bodies in the liver and muscle following administration of  $\beta$ -hydroxybutyric acid is found to be similar to that observed when ketosis is of endogenous origin.

5. The evidence indicates that the ketogenic effects of fasting, anterior pituitary extract, and phlorhizin are due to their action upon the metabolism of the liver cell, as a result of which there is

an increased ketone body production. The results further indicate that ketones are utilized by the muscle cell.

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## ESTROGENS WITH OXYGEN IN RING B

### III. 6-KETO- $\alpha$ -ESTRADIOL

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The conversion of equilin into 7-hydroxyestrone, and of the latter into  $\Delta_6$ -isoequilin has been described in Papers I and II of this series (1). An alternative route for the synthesis of estrogens possessing a double bond between carbon atoms 6 and 7 leads over derivatives substituted with oxygen in position 6. This pathway, however, was not explored beyond the stage of the last intermediate, the 6-hydroxy compound, because it is inferior to the synthesis already reported in regard to yields. The new estrogens obtained are nevertheless of interest, because they permit a comparison of the influence on physiological activity of a functional group in position 6 with the effect of a similar group in position 7. In the present communication we describe the preparation and properties of 6-keto- $\alpha$ -estradiol, and of an acidic by-product in which Ring B is partially degraded.

The introduction of a keto group in position 6 of estrone<sup>1</sup> or  $\alpha$ -estradiol can be accomplished by treatment of their acetates with chromic acid at room temperature. In our own studies, we preferred  $\alpha$ -estradiol diacetate as the starting compound, because both functional groups are protected and the points of attack thus limited. Furthermore, the material not attacked in the reaction can be readily separated from the ketonic reaction product by means of Girard's reagent, while in the case of estrone acetate,

\* Commonwealth Fund Fellow, 1938-39.

<sup>1</sup> The experiment with estrone acetate has been carried out by Dr. E. Schwenk of the Schering Corporation and will be reported in a separate communication.

which is itself ketonic, the separation has to be effected by fractional crystallization. About half of the weight of the starting compound is converted into acids by the treatment with chromic acid under the conditions employed; the neutral fraction was treated with Girard's reagent and yielded 20 to 25 per cent of the original weight in the form of ketones. From the non-ketonic

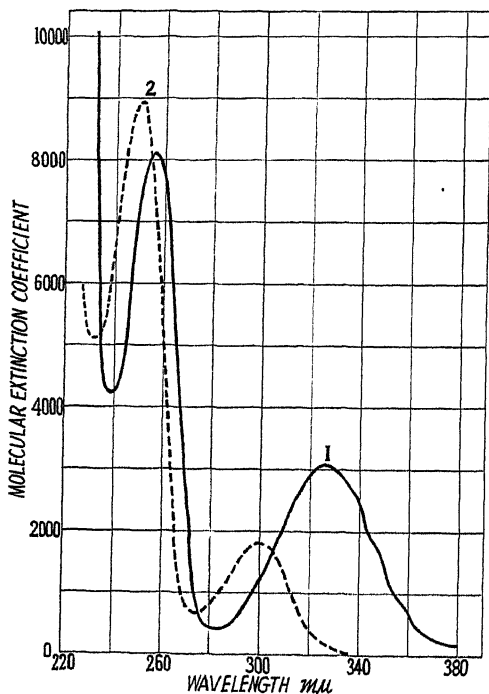


Fig. 1. Absorption spectra of 6-keto- $\alpha$ -estradiol (Curve 1) and of its diacetate (Curve 2).

portion some of the starting compound could be recovered. The ketonic fraction crystallized readily, but proved to be inhomogeneous. By fractional crystallization a substance melting at  $173^{\circ 2}$  could be separated, which according to the analysis was the expected diacetate of 6-ketoestradiol. The less soluble fractions which melted, with decomposition, over a range from

<sup>2</sup> All melting points reported in this paper are corrected.

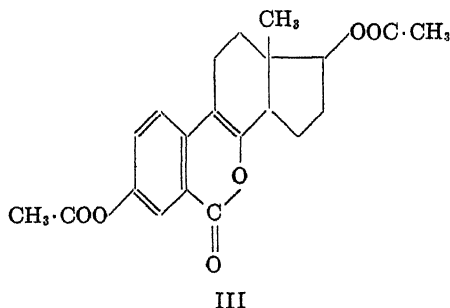
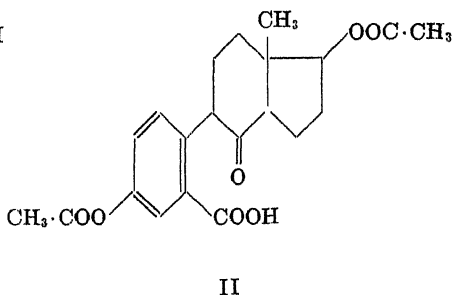
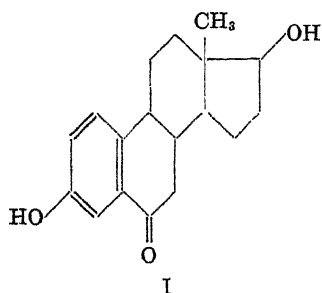
250–265°, failed to yield a pure compound on repeated recrystallization. Since in contradistinction to the diacetate this fraction coupled with *p*-nitrodiazobenzene, some hydrolysis had obviously taken place, probably during the splitting of the betaine hydrazone with hydrochloric acid. The high melting material was therefore hydrolyzed with alkali, when it readily yielded the free ketodiol, which melts at 283° (I). For later runs the whole ketonic fraction was hydrolyzed directly, or converted into the diacetate by re-acetylation. The ketonic function of the newly introduced oxygen atom was ascertained by the preparation of a semicarbazone. The characteristics of the ultraviolet spectrum (Fig. 1, Curve 1) clearly indicate that the keto group is situated in  $\alpha$  position to the phenolic ring. The extension of the conjugated system by the carbon-oxygen double linkage is evident in the emergence of a new band at 326  $m\mu$  ( $\epsilon = 3000$ ), while a more intense band at 256  $m\mu$  ( $\epsilon = 8000$ ) has appeared in place of the minimum occupying this region in the spectrum of estradiol. In the absorption curve of the diacetate (Fig. 1, Curve 2) the two maxima are shifted towards lower wave-lengths, an effect which generally attends the esterification of the phenolic hydroxyl groups in estrogens.

The specific rotation of 6-keto- $\alpha$ -estradiol in alcohol is +4°, that of  $\alpha$ -estradiol in the same solvent +81°. The marked decrease of dextrorotation brought about by the introduction of the 6-keto group is in accordance with the analogous case of testosterone ( $[\alpha]_D = +109^\circ$ ) (2) and 6-ketotestosterone ( $[\alpha]_D = -58^\circ$ ) (3).

On treatment with acetic anhydride in the presence of anhydrous sodium acetate only the two preformed hydroxy groups in 6-ketoestradiol are esterified, whereas under the same conditions the keto group in 7-ketoestrone reacts with the formation of a 7-enol acetate. In the latter case the enolization is obviously called forth by the tendency to extend the conjugated system by the addition of a 6-7 double bond. For the 6-ketone, on the other hand, the absence of enolization is ascribable to the fact that the keto group is already a part of the conjugated system.

The physiological potency of the 6-keto- $\alpha$ -estradiol was found to be 3 million rat units per gm. (estrone = 1 million rat units

per gm.).<sup>3</sup> Since with the method employed  $\alpha$ -estradiol consistently assayed 12 million rat units per gm., it follows that the introduction of the keto group has diminished the potency 4 times. The unfavorable effect of a keto group in position 6 is much less pronounced than that of one at carbon atom 7, whereby the physiological potency is decreased to about 1/300 of that of the parent estrogen.



The acidic fraction resulting from the treatment of  $\alpha$ -estradiol diacetate with chromic acid is a syrup of bright yellow color. In two instances material of this description when dissolved in a small volume of acetone deposited, on prolonged standing in the ice box, crystals which after purification melted sharply at 144–145°. The analytical composition  $C_{21}H_{24}O_7$  indicated that the two acetoxy groups were still present, but that 1 carbon atom had been lost in the course of the oxidation. This was borne out by

<sup>3</sup> The assay was carried out by Dr. C. Mazer of Philadelphia, to whom we wish to express our sincere thanks. The details of the method have been given in a previous publication (4).

a combined titration and saponification experiment which revealed one free carboxyl group and two saponifiable ester groups. These findings suggest that the compound is a keto acid possessing the structure (II). We assume that the first intermediate in the further oxidation of 6-ketoestradiol diacetate is a 6,7-diketone. Such a compound would show a strong tendency to enolize with the formation between carbon atoms 7 and 8 of a double bond which would furnish the preferential point of attack for the

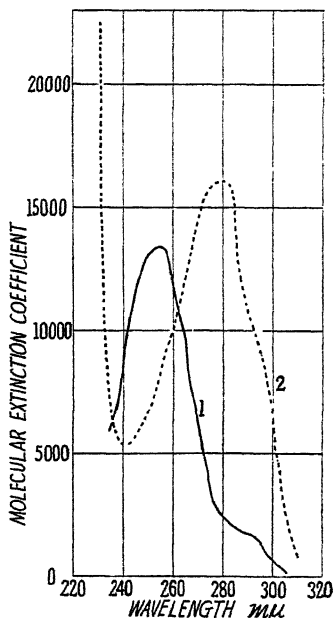


FIG. 2. Absorption spectra of the keto acid,  $C_{21}H_{24}O_7$  (Curve 1), and of its enol lactone (Curve 2).

following oxidative cleavage. The resulting diketone acid, in which the keto groups are derived from  $C_6$  and  $C_8$ , and the carboxyl group from  $C_7$ , would be decarboxylated immediately to (II). As will be pointed out below, very little if any dicarboxylic acids are present in the crude acidic fraction resulting from the oxidation with chromic acid. The above reaction mechanism, which postulates that the primary cleavage occurs between  $C_7$  and  $C_8$ , and not between  $C_6$  and  $C_7$ , provides an



explanation for this finding. If a 6,7-dicarboxylic acid were the first product resulting from the cleavage of Ring B, it should certainly be demonstrable in considerable quantity among the oxidation products, since there is no reason why such a structure should readily undergo oxidative decarboxylation to (II).

By treatment with hot acetic anhydride in the presence of anhydrous sodium acetate the acid is converted into a non-acidic crystalline compound which melts at 153°. Since, as the analysis shows, 1 molecule of water has been lost in its formation, it must be an enol lactone (III). The extension of the conjugated system by the new enolic double bond 8-9 should manifest itself in a shift of light absorption towards the red end of the spectrum. A comparison of the absorption curves of the keto acid and the lactone (Fig. 2) shows that this is indeed the case. The band at 254 m $\mu$  is displaced to 279 m $\mu$  and somewhat enhanced in height by the establishment of the enolic double bond. The spectrographic findings thus advance additional proof for the correctness of the structures assigned.

The yields of the keto acid which could be directly isolated from the acidic fraction varied a great deal in different runs. In one case this fraction refused altogether to crystallize. When non-crystalline material of this type was subjected to the reaction employed for the preparation of the lactone from the crystalline keto acid, by far the greater part of it was transformed into neutral products, from which the crystalline lactone could be isolated in excellent yield. This shows that the keto acid is the main acidic product formed when the oxidation progresses beyond the stage of the 6-ketone.

#### EXPERIMENTAL

*6-Keto- $\alpha$ -Estradiol Diacetate*—870 mg. of  $\alpha$ -estradiol diacetate, m.p. 126–128°, were dissolved in 3 cc. of glacial acetic acid, and a solution of 735 mg. of chromium trioxide (4.5 atoms of O) in 0.6 cc. of water and 4.4 cc. of glacial acetic acid added. The mixture was allowed to stand for 24 hours at 23–24°. The excess of chromic acid was then reduced with 1 cc. of ethanol. After dilution with water the reaction products were transferred into ether, and most of the acetic acid removed from the ether phase by distribution with portions of saturated sodium bicarbonate solution. The

point when acidic reaction products begin to react with the bicarbonate can be recognized by the pink color of the extract. The ether phase was then exhaustively extracted with a 3:1 mixture of 1 N sodium carbonate and saturated sodium bicarbonate solutions, washed with water, and brought to dryness with addition of absolute alcohol during the last stage of evaporation. The partly crystalline residue (436 mg.) was dissolved in 10 cc. of absolute alcohol and 1 cc. of glacial acetic acid; 1 gm. of Girard's Reagent T was added and the mixture boiled for 1 hour.

The separation of non-ketonic and ketonic fractions was carried out in the usual way; the splitting of the intermediate betaine hydrazone was effected by making the solution about 1 N in hydrochloric acid and allowing it to stand 2 hours at room temperature. The crystalline ketonic fraction (190 mg.) was re-acetylated by boiling for 20 minutes in 5 cc. of acetic anhydride. On decomposition of the reagent with 30 cc. of water a crystalline precipitate was obtained which was filtered off and washed with water (175 mg.). The rest of the material was recovered by extraction with ether from the filtrate (25 mg.). The two fractions were combined and recrystallized from absolute, then from 90 per cent alcohol. 149 mg. of needles melting at 173–175° were obtained. Acetylation of the pure ketodiol yielded the same compound.

Analysis— $C_{22}H_{26}O_5$ .	Calculated.	C 71.31,	H 7.08
	Found.	" 71.00, 71.27,	" 7.15, 7.03

*6-Keto- $\alpha$ -Estradiol*—As mentioned previously, the ketonic fraction resulting from the separation with Girard's reagent consists of a mixture of the diacetate and partially saponified products. On recrystallization from absolute alcohol 187 mg. of such a mixture yielded 126 mg. of material which melted with gradual decomposition over a range from 215–260°, after some sintering at 165°. From the mother liquors a small amount of the diacetate, melting at 173°, could be isolated by fractional crystallization. The higher melting material was dissolved in 3.5 cc. of 20 per cent methyl alcoholic potassium hydroxide. The yellow solution was allowed to stand 24 hours at room temperature in a nitrogen atmosphere. It was then diluted with water, acidified with hydrochloric acid, which caused the color to disappear, and extracted three times

with ether. The ether layer was washed with 5 per cent sodium carbonate solution saturated with sodium bicarbonate, and with water. (Sodium carbonate alone appeared to extract some ketodiol, since the solution became strongly yellow.) The almost colorless crystalline residue of the ether solution (96 mg.) was recrystallized twice from absolute alcohol. The pure compound crystallizes in rather large hexagonal plates melting at 281–283° with slight decomposition. Saponification of the pure diacetate yielded a product with identical properties.

*Analysis*— $C_{15}H_{22}O_3$ . Calculated. C 75.48, H 7.75

Found. " 75.33, " 7.67

$[\alpha]_D^{25} = +4.2^\circ$  (0.7% in alcohol)

The solution of 6-keto- $\alpha$ -estradiol in concentrated sulfuric acid is greenish yellow, but shows no fluorescence. On dilution with water the color changes to a deep purple with bluish fluorescence. On coupling with diazotized *p*-nitroaniline in alkaline solution the compound forms a dark red pigment, which becomes brick-red on acidification with acetic acid. When the ketodiol or its diacetate is dissolved in aqueous or methyl alcoholic potassium hydroxide, the solution immediately assumes a yellow color, which disappears on acidification. In contradistinction to  $\alpha$ -estradiol the ketone is not precipitated by digitonin in 80 per cent ethanol.

The semicarbazone was prepared from 8.6 mg. of the ketodiol with an excess of semicarbazide acetate in 90 per cent methanol at room temperature. After 40 hours the crude product was precipitated with water and after washing and drying weighed 11 mg. Two recrystallizations from a small volume of absolute alcohol yielded 6 mg. of needles which decomposed at 280–310° with gas evolution.

*Analysis*— $C_{15}H_{25}O_3N_3$ . Calculated, N 12.24; found, N 12.02

*Diacetoxy Keto Acid*,  $C_{21}H_{24}O_7$ —The alkaline extracts containing the acidic oxidation products were acidified with hydrochloric acid and extracted repeatedly with ether. The ether extracts were washed thoroughly with water, and dried with sodium sulfate. The ether residue (480 mg.) was liquefied with about 1 cc. of acetone. After several days standing in the refrigerator a mass of crystals had formed. The remaining oil was washed away with

small volumes of cold acetone and the crystalline product (223 mg.) recrystallized twice from 80 per cent alcohol. The acid crystallizes in large beautiful rods with oblique ends. The crystals retained a slight amount of a yellow pigment which could not be removed by recrystallization from alcohol or benzene-petroleum ether. That this color is not a property of the substance itself is proved by its disappearance on conversion of the acid to the enol lactone. Since in the latter the conjugated system is extended by one more double bond, the color should become more intense if a chromophoric group absorbing in the visible range were already present in the acid. Actually, however, the intensification of light absorption consequent on the reaction mentioned is confined to the ultraviolet region.

<i>Analysis</i> — $C_{21}H_{24}O_7$ .	Calculated.	C 64.92,	H 6.23
	Found.	" 65.24, 65.26,	" 6.31, 6.46

97.8 mg. of the acid were dissolved in 5 cc. of absolute alcohol and titrated with standard alkali and phenolphthalein as indicator. 2.35 cc. of 0.1 N NaOH were neutralized (calculated 2.51 cc.). More alkali was added, and the excess titrated back after 20 hours standing under nitrogen. 4.44 cc. of 0.1 N NaOH, calculated for two hydrolyzable groups, 5.02 cc. The end-points in both titrations were somewhat blurred, as a scarlet red pigment developed in alkaline solution. This difficulty is probably responsible for the low titration values. The completely hydrolyzed acid was recovered by acidification and ether extraction, but resisted all attempts at crystallization. Increasing pigmentation during manipulation in hot solvents indicates a marked instability of the hydrolyzed product, whereas the diacetoxy acid showed no such behavior.

In an attempt to prepare the semicarbazone of the crystalline acid an amorphous substance soluble in alcohol and ether was obtained in unsatisfactory yield. Treatment of the acid with diazomethane results in the formation of the methyl ester, which, however, failed to crystallize.

*Enol Lactone*,  $C_{21}H_{22}O_6$ —To 22.5 mg. of the keto acid 1 cc. of acetic anhydride and 150 mg. of anhydrous sodium acetate were added. The mixture was boiled for 1 hour. On decomposition of the reagent with ice-cold water a part of the reaction product

deposited in crystalline form. Without being filtered from the latter, the supernatant was carefully neutralized to litmus with sodium hydroxide. An excess of alkali rapidly hydrolyzes the lactone. The aqueous phase was extracted several times with ether. The combined ether solutions were washed once with dilute sodium bicarbonate solution and with water. Recrystallization of the ether residue (18 mg.) from 80 per cent alcohol yielded colorless rhombohedral plates which melted at 152–153°.

*Analysis*— $C_{21}H_{22}O_6$ . Calculated. C 68.09, H 5.99  
Found. " 68.35, 68.33, " 6.44, 6.09

In the preparation of the lactone from uncrystallizable acidic fractions from the chromic acid oxidation the neutralization with sodium hydroxide was omitted, and the ether phase extracted instead three times with half saturated sodium bicarbonate solution. About 700 mg. of the original acidic material yielded 139 mg. of uncrystallizable, strongly pigmented acids and 525 mg. of neutral products. From the latter 294 mg. of crystals were isolated. An additional crop was obtained from the strongly pigmented mother liquors which were brought to dryness, dissolved in absolute ether, and decolorized with charcoal. After two crystallizations from absolute alcohol the crystals melted at 152–153°.

Attempts to reconvert the enol lactone, which thus can be obtained in large quantities, to the less easily accessible keto acid met with failure. Cautious titration with alkali showed rapid liberation of an acidic group in the initial stage, but after addition of 1 mole of alkali less than half of the material had been converted into acidic products not extractable with ether from sodium carbonate solution. Both the acidic and neutral fraction from this distribution failed to crystallize. Apparently partial hydrolysis of the phenolic acetoxy group had occurred to about the same extent as opening of the lactone ring, so that mixtures were obtained in both fractions.

Hydrolysis of the lactone with boiling methyl alcoholic hydrochloric acid yielded mainly acidic products which failed to crystallize and became pigmented on standing.

#### SUMMARY

The preparation of 6-keto- $\alpha$ -estradiol by treatment of  $\alpha$ -estradiol diacetate with chromic acid is described. The introduction of

the keto group diminishes the estrogenic potency to one-fourth of that of  $\alpha$ -estradiol.

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# THE LIPID CONTENT OF BLOOD, LIVER, AND YOLK SAC OF THE NEWLY HATCHED CHICK AND THE CHANGES THAT OCCUR IN THESE TISSUES DURING THE FIRST MONTH OF LIFE\*

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The features that distinguish the lipid metabolism of the bird from that of the mammal were pointed out in recent reports from these laboratories. In the bird, maturity brings with it an increased content of lipids in both blood and liver (1, 2), and these changes were shown to be the result of *estrogenic activity* (3, 4). In the course of these investigations, birds between the ages of 59 and 299 days were studied. At the present time a survey of the entire life cycle is under investigation. The present report deals with the period between hatching and 36 days of age. It is shown that the bird enters the external world with a lipemia and a fatty liver. *The liver of the newly hatched bird contains enormous amounts of cholesterol, the major part of which is in the esterified form.*

Although high lipid levels in the tissues of the chick embryo have been recognized for some time, the lipid metabolism of the bird during the period studied here has received very little attention. A few observations dealing with the blood fat (5) and blood cholesterol (6) of 1 and 2 day-old chicks have appeared, but the values reported are not in accord with the findings obtained in the present investigation.

\* This investigation was supported in part by a grant from the Committee on Research in Endocrinology of the National Research Council. Assistance was also furnished by the Works Progress Administration (Official Project No. 65-1-08-62, Unit A6).



## EXPERIMENTAL

Chicks were obtained from eggs produced by the single comb white Leghorn flock of the Poultry Division. They were hatched in incubators of the forced draft cabinet type with separate hatching compartments. On the 22nd day after the eggs were placed in the incubators, all healthy chicks were transferred to battery brooders kept at constant temperature, and thereafter the chicks had access to food and water. It has been noted that little or no food is eaten by chicks during the first 24 hours after hatching.

The average weights of the chicks taken for analyses are recorded in Fig. 1. Liver, blood, and yolks were pooled for analyses. No attempt was made to separate the yolk sac from its contents. Lipids were determined on the entire structure.

The data shown below were obtained from three separate experiments. In two of these blood, liver, and yolk sacs were obtained, in the third only blood.

The microoxidative procedures employed for lipid analyses of blood and liver have been recorded elsewhere (1, 2). The yolks were treated like the liver.

*Liver Lipids*

The data shown in Fig. 2 represent the mean values obtained from two separate experiments.

*Total Lipid*<sup>1</sup>—In Experiment 1, the liver of the newly hatched bird contained from 13 to 14.7 per cent of total lipid, whereas in Experiment 2 values as high as 23 per cent were found immediately after hatching. This high lipid content did not remain for long, for in Experiment 1 a fall had already occurred by the 4th day and despite irregularities the loss of lipids from the liver became quite definite by the 6th day. Between 5.0 and 5.6 per cent of total lipids was found in the 8 day-old chick, and little change was observed during the remainder of the 16 days of Experiment 1. In Experiment 2, several chicks were also examined on the 22nd and 36th day after hatching. In these pools, values for total lipid between 1.6 and 3.2 per cent of the wet weight of the liver were noted.

<sup>1</sup> Calculated as the sum of total fatty acids and total cholesterol.

*Cholesterol*—Enormous concentrations of cholesterol are present in the liver of the newly hatched chick. During the first 3 days the chick livers in Experiment 1 contained from 5.9 to 8.5 per cent of total cholesterol. In Experiment 2, the values were even higher; the maximum observed was 9.7 per cent. Cholesterol at this time may account for as much as 48 per cent of the total lipids. From the 3rd day on, the decline in the cholesterol

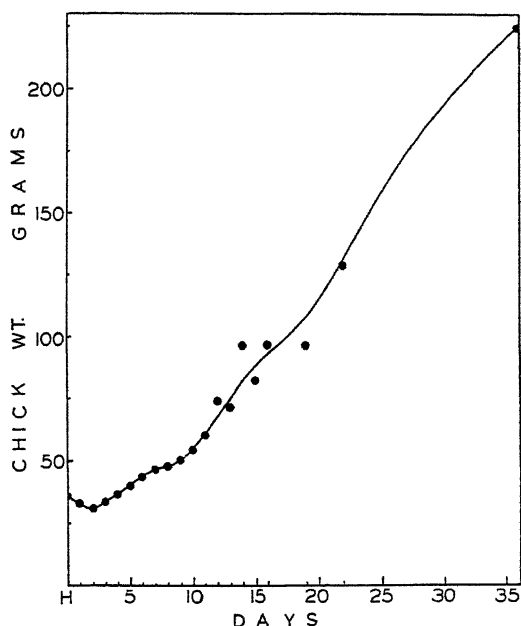


FIG. 1. The growth of the chicks used in this investigation, as shown by the average weights of the birds taken for analyses. Day H is the day of hatching.

content is rapid. In the 9 day-old chick the total cholesterol content of the liver had fallen to approximately 1 per cent, while values close to 0.5 per cent were found in the 11 day-old chick. The 22 day- and 36 day-old chick livers contained between 0.3 and 0.4 per cent of total cholesterol; these values approximate closely those found in the livers of adult birds.

The major portion of the cholesterol was in the esterified form during the early days after hatching. Values as high as 1.2 per

cent were found for free cholesterol; high values were still present as late as 3 days after hatching in Experiment 1 and as late as 5 days in Experiment 2. On the day of hatching esterified cholesterol was present to the extent of 5.4 to 5.9 per cent in the livers in Experiment 1, whereas in Experiment 2 values of 6.6 to 8.6 were found. In the 7 day-old chick the content of esterified cholesterol in the liver was still above 1 per cent. Values as low

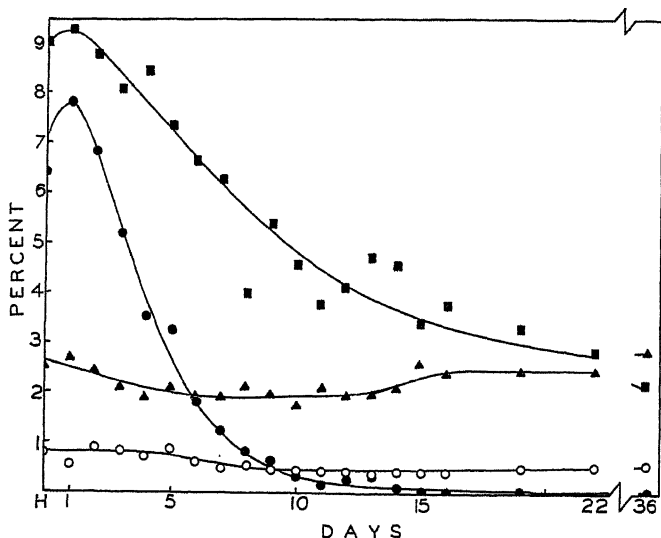


FIG. 2. Liver lipids of the chick from the day of hatching (Day H) to 36 days of age. ■ total fatty acids, ● esterified cholesterol, ○ free cholesterol, ▲ phospholipids. The values are expressed as the per cent of the wet weight of tissue. Each point represents the average values obtained from two to five pools, each of which contained from two to four livers, the number used depending upon the weight of tissue obtained. More pools were taken from younger than from older chicks.

as those found in the mature bird do not appear until the chick attains an age of 14 days or more.

*Total Fatty Acids*—The liver of the chick is particularly rich in fatty acids during the first 7 days after hatching. In Experiment 1, the highest value, namely 10.4 per cent, was found in 4 day-old chicks, but in Experiment 2 values of 14.0 and 12.7 per cent were noted during the first 24 hours after hatching. High values may still be present as late as 7 days after hatching, although—to

judge by the average values (Fig. 2)—the liver loses fatty acids rapidly during the first 7 days after hatching. Fatty acids continue to be lost thereafter, but the rate at which this occurs is much slower and more irregular between the ages of 7 and 22 days than between the time of hatching and the age of 7 days.

*Shift in Liver Fatty Acids from Cholesterol Esters to Triglycerides during Early Days after Hatching*—The fatty acids combined as phospholipid, triglycerides, and cholesterol esters have been calculated for the chick liver and shown in Fig. 3. Phospholipid fatty acids remained essentially constant throughout. Between

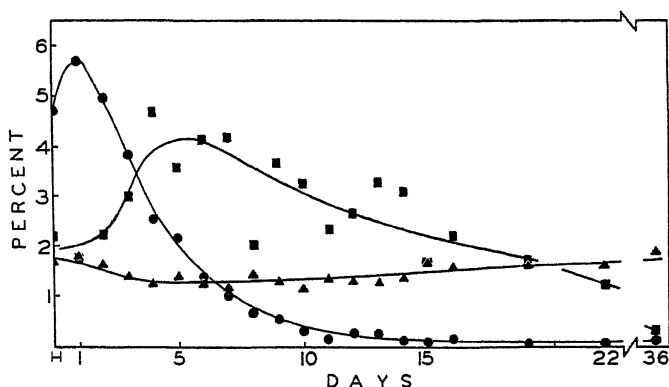


FIG. 3. Liver fatty acids present as triglycerides, cholesterol esters, and phospholipid. ● cholesterol ester fatty acids (1), ▲ phospholipid fatty acids (2), ■ neutral fat fatty acids (3). These were derived as follows: (1)  $0.73 \times$  esterified cholesterol; (2)  $0.67 \times$  phospholipid; (3) total fatty acids minus (cholesterol fatty acids + phospholipid fatty acids). Day H is the day of hatching.

the 2nd and 7th day after hatching, a rise in the triglyceride fatty acids was found in the liver. This occurs simultaneously with a rapid loss of cholesterol fatty acids. Apparently, during the utilization of cholesterol esters in the liver, the fatty acid portion is converted to triglycerides. It can be observed from Fig. 2 that the various lipid constituents do not fall at the same rate; this shift in fatty acids from cholesterol esters to triglycerides is in keeping with the finding of a more precipitous fall in esterified cholesterol than in total fatty acids.

*Phospholipids*—This lipid constituent showed no major change

throughout the period studied. Thus in Experiment 1 phospholipids were present to the extent of 1.6 to 2.5 per cent in the

TABLE I  
*Blood Lipids of Chick*

Experiment No.	Age of chick	Blood sample		Cholesterol			Total fatty acids	Phospholipids	Total lipids	Residual fatty acids*
		Volume	Chicks used	Total	Free	Esterified				
	days	cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	H†	4	12	284	152	132	666	445	950	271
	1	7	12	314	147	167	866	486	1180	418
	3	6	12	289	159	130	721	337	1010	400
	4	10	12	224	137	87	485	326	709	203
	5	10	12	220	121	99	450	314	670	167
	6	10	12	191	119	72	461	274	652	225
	7	10	12	178	108	70	302	326	480	32
	8	10	12	154	108	46	382	366	536	103
2	H†	5	8	302	172	130	560	410	862	190
	1	5	8	370	338	32	768	384	1138	487
	2	5	8	422	218	204	624	450	1046	174
	2	5	8	387	197	190	619	320	1006	266
	3	5	8	446	220	226	654	442	1100	193
	5	7	6	296	160	136	458	310	754	151
	6	7	6	235	147	88	622	214	857	414
	8	7	6	143	115	28	506	472	649	169
	15	8	6	126	101	25	326	281	452	119
	22	10	4	111	79	32	342	255	453	150
	36	10	4	110	98	12	451	265	561	262
3	H†	5	8	326	177	149	615	446	941	204
	1	5	9	366	205	161	708	469	1074	276
	2	5	7	344	167	177	780	474	1124	333
	3	5	7	288	162	126	727	437	1015	342
	4	5	6	323	161	162	610	392	933	229
	5	5	4	291	154	137	571	497	862	138
	6	5	6	220	119	101	676	334	896	378

\* Mainly neutral fat fatty acids (see the legend of Fig. 3).

† Day of hatching.

livers of the newly hatched chicks, whereas in the 16 day-old bird values of 2.2 to 2.4 per cent were found. The 36 day-old bird contained 2.7 to 2.8 per cent phospholipid.

*Blood Lipids*

The lipid concentration of the blood is particularly high during the first 4 days after hatching (Table I). Values for total lipid well over 1000 mg. per 100 cc. of whole blood were found during this interval. By the time the chicks reached an age of 15 days, the level of the total lipids in the blood fell to 452 mg. On the day the chicks were hatched, values of 284 to 326 mg. were observed for total cholesterol, while in the 8 day-old chick it had dropped to 143 to 154 mg. As much as 50 per cent of the cholesterol was in the esterified form during the early days but the ratio of ester to total cholesterol fell as the chick grew older. Phospholipids were also present in increased amounts during the early days after hatching. Values between 410 and 446 mg. were found on the day of hatching.

*Yolk Sac*

The yolk sac is a diverticulum of the small intestine and weighs approximately 5 gm. in the newly hatched chick. Yolk material is absorbed directly from the sac into the blood stream. Absorption proceeds rapidly after the bird is hatched, and normally by the 3rd day the weight of the sac is reduced to about 1 gm. The absorption of this structure is practically complete 5 days after the chick has hatched, but small remnants of the sac often persist much longer.

The content of cholesterol, phospholipid, and fatty acids, measured as percentage of the wet weight of this organ, is recorded in Fig. 4. The yolk sac at the time of hatching contained 11 per cent of fatty acids (Fig. 4) or a total of about 0.5 gm. (Figs. 4 and 5). The other lipid constituents were present in much smaller amounts. At the time of hatching, the entire yolk sac contained about 0.07 gm. of phospholipid and about 0.06 gm. of total cholesterol. So rapidly does the absorption of fatty acids proceed that, by the 5th day after the chick has hatched, less than 0.03 gm. of this lipid constituent remains in the entire yolk sac. The loss in fatty acids occurs not only in absolute amounts contained in the entire sac but also in the amount present as percentage of the wet weight of the organ. The loss in cholesterol

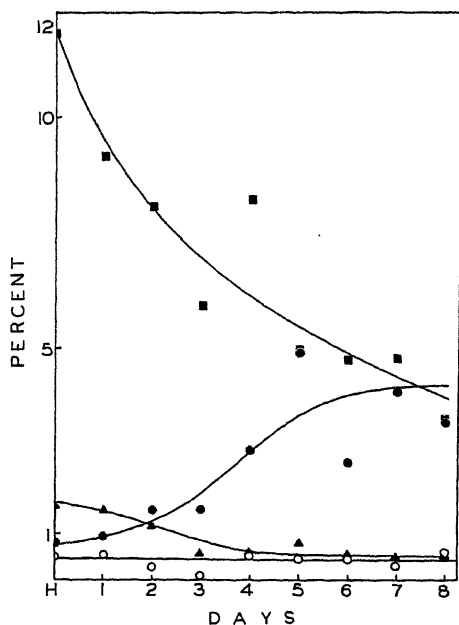


FIG. 4. The lipids of the yolk sac from the day of hatching (Day H) to 8 days of age. ■ total fatty acids, ● esterified cholesterol, ○ free cholesterol, ▲ phospholipid. The values are expressed as per cent of the wet weight of the yolk sacs.

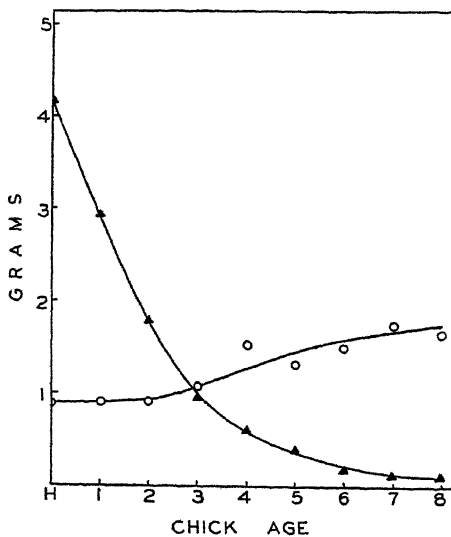


FIG. 5. The average weights of the yolk sacs and livers from the day of hatching (Day H) to 8 days of age. ▲ yolk sac, ○ liver.

and phospholipid occurs in a less striking manner. Little or no change was observed in free cholesterol during the first few days after hatching when this lipid constituent was measured as percentage of the wet weight of the yolk sac, but some loss in absolute amount did occur, since this organ was rapidly shrinking in size. Phospholipids decreased in both the total amount present and the amount measured as percentage of the wet weight of the yolk sac. The cholesterol esters of the yolk sac showed decreases in absolute amounts, but when the amounts of this lipid constituent were expressed as percentage of the organ, it was noted that a rise had occurred (Fig. 4). This suggests that the removal of cholesterol esters from the yolk sac is proceeding less rapidly than that of other constituents.

#### DISCUSSION

The chick emerges from the egg with large deposits of lipids in three tissues, liver, yolk sac, and blood. The rapid loss of these lipid stores affords an opportunity for comparing the utilization of the various lipid constituents of these three tissues. Phospholipids, cholesterol, and triglycerides (neutral fat) do not leave the tissues at the same rate. Triglycerides are most rapidly removed from the yolk sac, and this results in a relative—though not absolute—increase in the percentage of ester cholesterol. The liver behaves differently. In this tissue, esterified cholesterol is most rapidly removed. The fatty acids combined with cholesterol are not as rapidly removed but reappear temporarily as triglycerides in the liver. Thus at a time when the chick's liver is being rapidly depleted of its large store of cholesterol, an absolute increase in neutral fat is found in the liver, an observation that suggests that esterification serves merely for storage of cholesterol.

The mechanism by which the fatty liver and lipemia are produced in the newly hatched chick remains to be considered. During its development the chick is nourished by the yolk, which is unusually rich in both fat and cholesterol. A rough parallel may be observed between the time of disappearance of the yolk sac after hatching and the time of disappearance of excessive amounts of fat from both blood and liver (compare Figs. 2 and 5). This suggests that the high content of cholesterol in the yolk



contributes to the fatty liver and lipemia found in the chick. Okey and Yokela (7) reported that rats fed egg yolk or purified cholesterol for as long as 60 days had fatty livers containing as much as 26 per cent fatty acids and 8 per cent cholesterol, over 90 per cent of which was esterified. The striking similarity between this type of fatty liver and that observed in newly hatched chicks lends support to the view that cholesterol is responsible for the fatty liver of the chick. Still another factor may play a part in the production of the high lipid content of the chick tissues. It has been reported by several investigators (8-10) that appreciable quantities of estrogens are present in the yolk. It was previously observed in these laboratories that the injection of estrogens in immature birds results in a lipemia similar to that found normally in laying birds (4); the estrogens present in the egg yolk may thus be responsible for the lipemia of the baby chick. A fatty liver is also observed in the laying bird (2). But, judging from the distribution of the various lipid constituents in the liver, the fatty liver of the baby chick resembles more closely the fatty liver of the cholesterol-fed rat.

#### SUMMARY

The lipid content of the blood and liver of the chick was investigated during the first 36 days after hatching.

1. The liver of the newly hatched chick contained an average of 7.3 per cent cholesterol (90 per cent of which is esterified) and 9.0 per cent total fatty acids.

2. The blood of the newly hatched chick contained as much as 1000 mg. of total lipid per 100 cc. of whole blood. All lipid constituents, namely cholesterol, total fatty acids, and phospholipids, were present in amounts greater than in the blood of the mature male and mature non-laying female bird.

3. A rapid decline in the lipid content of blood and liver set in on the 3rd day after hatching. The rapid fall in esterified cholesterol in the liver was associated with a temporary rise in triglycerides.

4. When the chicks were hatched, the yolk sacs contained about 12 per cent fatty acids, 1.6 per cent phospholipid, and 1.3 per cent total cholesterol. Their absorption proceeded rapidly and was

nearly complete by the time the chicks were 5 days old. Neutral fat was absorbed more rapidly than the other lipid constituents.

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## PREPARATION AND ASSAY OF ANTERIOR PITUITARY FRACTIONS RICH IN KETOGENIC AND RESPIRATORY QUOTIENT-REDUCING SUBSTANCES\*

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A number of metabolic effects have been attributed to anterior pituitary extracts and various criteria have been proposed for the determination of the relative potency of different preparations. Extracts have been tested for their effects on (a) ketonuria in the fasted and fed animal on various diets (1-6), (b) ketonemia (1, 2, 7-10), (c) antagonism to the hypoglycemic action of insulin (11), (d) hyperglycemic action (12), (e) diabetogenic action (13), (f) glycostatic effects (14), (g) ability to increase liver fat in various species (3, 6, 15), (h) increase oxygen consumption (16-19), (i) alter nitrogen metabolism (20), (j) depress the level of blood fat (21), and (k) depress the respiratory quotient (22). In view of the apparent interdependence of fat, carbohydrate, and protein metabolism, and of the numerous physiological effects observed with pituitary extracts, it would seem probable that some of these effects are only different manifestations of the metabolic influences of the same factor. With this view as a working hypothesis, we have been attempting for several years to fractionate anterior lobe extracts with the object of separating in a single fraction the factor or factors which cause these metabolic effects. Progress has been slow, first because of the apparent

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lability of the active substance, and second because of the lack of dependable criteria for quantitative measurement of activities.

Believing it essential to have methods for quantitative assay of activity, we decided to concentrate efforts on the attempt to standardize conditions and technique for measuring at least one effect. The influence of the extracts upon the respiratory quotient of glucose-fed rats was chosen as the basis for study. Simultaneously another study was made by one of us (I. K. F.) of the measurement of the ketonuric and ketonemic effects in rats. With these criteria for semiquantitative assay of two metabolic effects, fractionation of the extracts has led to a concentration in the same fraction of the active principle responsible for both these effects. These fractions are relatively free from other known hormones (except the growth substance).

*Method of Assay for the Respiratory Quotient Factor*—Young male rats<sup>1</sup> of body weights of 150 to 250 gm. were maintained on a Purina Dog Chow diet<sup>2</sup> supplemented with cod liver oil and lettuce twice weekly. After being fasted 20 hours the animals were injected intraperitoneally with either saline or anterior pituitary preparations. The volume of injected material in the earlier experiments varied from 0.1 to 4.0 cc. In later series a constant volume of 2.0 cc. (of the desired dilution) has been used in all cases. 2 hours after the injection the rats were given 700 mg. of glucose per 100 gm. of body weight by stomach tube and were immediately placed in the animal chamber of the respirometer which was then ventilated for 5 minutes with dry, CO<sub>2</sub>-free air. The animal chamber was disconnected, closed, and weighed (to 5.0 mg.). The respiratory metabolism was then determined<sup>3</sup> during the subsequent 3 hour period, the stream of air through the system being adjusted at a rate (about 24 liters per hour) to prevent condensation of moisture on the sides of the animal chambers. At the end of the period the animal chamber, as well as the absorption flasks, was again weighed and the value corrected for temperature changes. The temperatures in the respiratory chamber varied from 28° to 33° on different days,

<sup>1</sup> Kindly supplied by Anheuser-Busch, Inc.

<sup>2</sup> Protein 23 per cent, fat 5.0 per cent, carbohydrate (by difference) 67 per cent.

<sup>3</sup> The gravimetric procedure of Haldane was employed.

within which limits consistent results were obtained. At higher temperatures the data were variable and unsatisfactory. The efficiency of the water absorbers ( $\text{H}_2\text{SO}_4$ ) was checked from time to time by introducing magnesium perchlorate bottles into the system. Two  $\text{CO}_2$  absorbing units were used. The first contained Wilson (8 mesh) soda lime and  $\text{H}_2\text{SO}_4$ . The second unit contained soda lime and calcium chloride, and served as a check on the efficiency of the first.

R.Q. values after glucose feeding and saline injection only (controls) varied from 0.81 to 0.97. The values obtained from a given animal under comparable conditions on different occasions were quite consistent. Inasmuch as the assay method is based on the depression of the R.Q. below the control level caused by the injection of pituitary extracts, it is necessary to use only rats giving high control R.Q. values. The plan was therefore adopted of making initial control determinations on all animals; those giving values below 0.86 (arbitrarily chosen) were discarded. About three-fourths of all the animals tested gave control values at or above this level.

The animals giving acceptable R.Q. values were then used repeatedly at weekly intervals either for the assay of anterior pituitary preparations or for additional control R.Q. determinations. It soon became apparent that some animals after several weeks use gave abnormally low respiratory quotients when tested with glucose and saline only and that the number of animals showing these (acquired) low control responses increased with the number of injections of pituitary extract, 20 to 40 per cent of a given group being affected after ten to fifteen weekly tests.<sup>4</sup> Fig. 1 illustrates the progressive decrease in the control R.Q. values in some individual animals injected with anterior pituitary extract for the periods of time shown on the abscissa. This difficulty was avoided by selecting animals giving control R.Q. values over 0.86, using them for four or five weekly assay tests, and then discarding them. With these precautions the results have been consistent and it is believed that the method is suitable for an approximate assay of activity.

The extent of depression of the R.Q. below the control values has been used as an index of activity of the material administered.

<sup>4</sup> The cause of these abnormal responses is being studied further.

The results of the assay of different anterior pituitary preparations at various dosage levels are compared by plotting the R.Q. values against the quantity of extract administered. Having thus determined the amount of a given extract needed to depress the R.Q. to 0.80, we calculated the multiples of this dose administered at other dosage levels. The composite curve obtained from a number of extracts is shown in Fig. 2. A unit of material

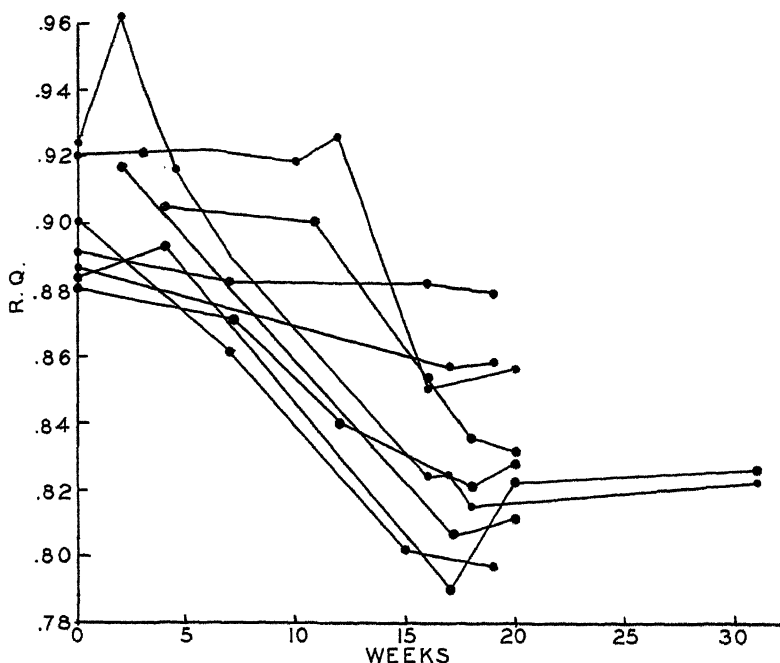


FIG. 1. The effect of repeated weekly injections of anterior pituitary extract upon the control R.Q. response of the glucose-fed rat.

is arbitrarily defined as that amount which when injected into animals under standard conditions will depress the R.Q. values to 0.80. Between the limits of 0.2 and 2.0 units the curve can be used for the evaluation of the potency of extracts. When the R.Q. values of the data in Fig. 2 are plotted against the log of the dose, the curve approximates a straight line between the dosage levels 0.2 to 2.0 units (see Fig. 3). Thus, the unit dose may be determined by injecting two series of animals at two dos-

age levels between 0.2 and 2.0 units. Consistent assay values have been obtained in this way by the use of four to eight animals per point.

*Preparations of Anterior Pituitary Extracts*—Beef pituitaries were used in all cases. The glands were packed in dry ice at the slaughter-house and stored at  $-10^{\circ}$  until the anterior lobes were dissected. All procedures have been carried out in the cold room at approximately  $0^{\circ}$ . Various methods of extraction have been

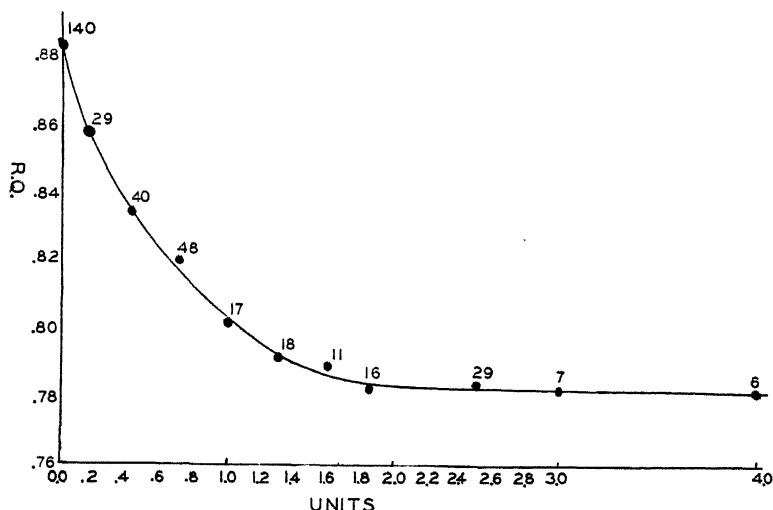


FIG. 2. Curve showing the relationship between R.Q. reduction and amounts of anterior pituitary extract administered. The small figures on the curve indicate the number of determinations used to evaluate the point.

tried. Extraction by alkaline solutions has invariably given better yields than either by acid or neutral solutions. Alcoholic extraction has failed consistently. Extraction with  $\text{Ba}(\text{OH})_2$  or  $\text{Ca}(\text{OH})_2$  by a slight modification of the Evans, Cornish, and Simpson method (23) has given the most satisfactory results. Complete centrifugation and rigid control of the salt concentration are essential to the success of the method.

The quantity of ground glands used in the preparation of different extracts has varied from 100 to 600 gm. A typical



extraction was carried out as follows: 5 volumes of cold distilled water were added to the glands and the solution was stirred until homogeneous (20 to 45 minutes). 0.2 N  $\text{Ba}(\text{OH})_2$  solution was slowly added (with stirring) until the solution reached pH 11.0 (glass electrode). This required about 1 volume of base. The mash was stirred for 10 to 15 minutes and then stored in a stoppered flask at  $0^\circ$  in an ice bath. The extraction was carried out for 4 days, the pH being maintained at or near 11.0 by frequent additions of small amounts of base (see the accompanying diagram). The mash was then strained through cheese-cloth

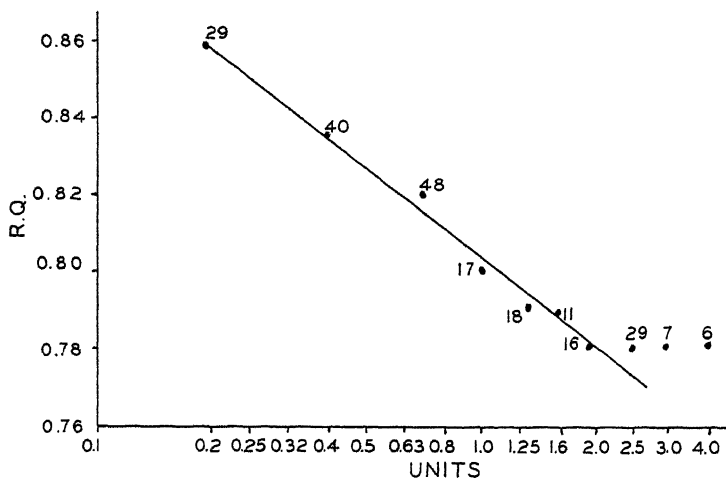
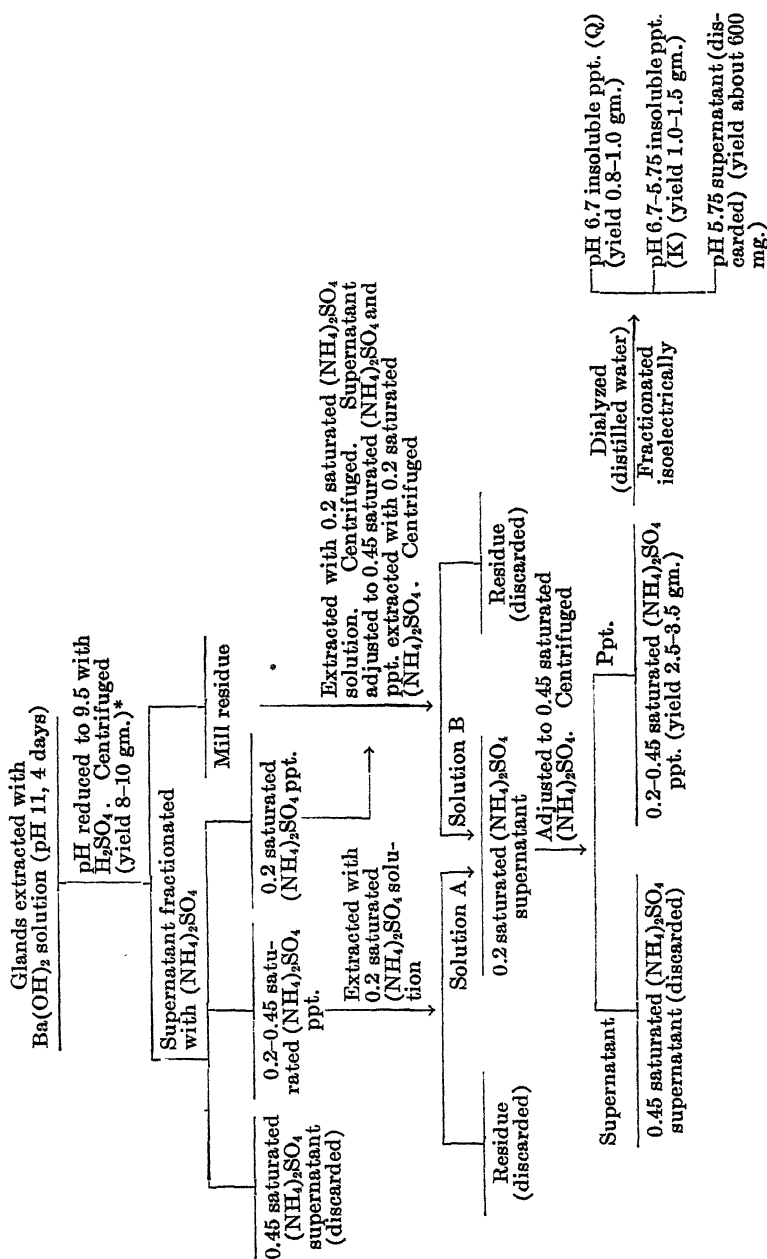


FIG. 3. Same data shown in Fig. 2 plotted on a semilogarithmic scale.

and the pH reduced to 9.5 by the *slow* addition of 0.2 N  $\text{H}_2\text{SO}_4$ .<sup>5</sup> No  $\text{Na}_2\text{SO}_4$  was added to the solution. The solution was passed through a Sharples centrifuge repeatedly until clear. Solid ammonium sulfate was added to make 0.2 saturation (12 gm. per 100 cc. of solution). After standing 20 minutes the solution

<sup>5</sup> When the pH of the extract was reduced to 7.0 to 8.0 soon after the extraction process was initiated, marked loss of activity often resulted. Extracts previously stored at pH 11.0 for a number of days were more stable in the region of pH 7.0 to 8.0. We are inclined to attribute this stabilization to the destruction of proteolytic enzymes by the alkaline solutions.



\* All yields are calculated as protein per 100 gm. of fresh glands.

was centrifuged. The supernatant liquid was brought to 0.45 saturation by the addition of 15 gm. of solid ammonium sulfate per 100 cc. of solution. After the salt dissolved, the mixture was allowed to stand 20 minutes, when the precipitate was removed by centrifugation (Sharples). The precipitate was next extracted with 6 volumes (volume equal to initial volume of solution) of 0.2 saturated cold ammonium sulfate solution. This solution (A) was allowed to stand 1 hour; during this time all solid residues from the initial extraction were reextracted with 3 volumes of 0.2 saturated ammonium sulfate solution. The suspension was centrifuged and the supernatant adjusted to 0.45 saturation as above. The precipitate was removed and extracted with 1.5 volumes of 0.2 saturated ammonium sulfate (Solution B). Solutions A and B were combined, centrifuged, and the salt concentration then adjusted to 0.45 saturation and recentrifuged. The total precipitate was placed in collodion or Visking casings and dialyzed in a mechanical dialyzer for 60 hours at 0° against large volumes of distilled water. The total procedure from the time the pH was adjusted to 9.5 to the dialysis step was carried out during the course of 1 day, as it was found that storage of extracts in the ice box overnight at intermediate stages often resulted in marked loss of activity.

The dialyzed material (pH 6.7 to 7.0) was centrifuged, and the precipitate was extracted with 1 volume of distilled water and recentrifuged. This was marked Precipitate Q. The above two supernatants were combined, the pH adjusted to 5.75 by means of 0.2 N HCl, and the resulting precipitate removed by centrifugation. This was marked Precipitate K. Precipitates K and Q were spread in thin layers each on hard filter paper and dried in a vacuum desiccator containing  $\text{H}_2\text{SO}_4$  and an excess of dry ice.<sup>6</sup> Both fractions proved to be rich sources of the r.q.-depressing factor. Approximately 5000 to 6000 r.q.-depressing units per 100 gm. of anterior lobe material were obtained by this method. This represents about 40 per cent of the active material present in the original crude extract. These powders are soluble in alkaline or neutral salt solutions; they are less

<sup>6</sup> Later preparations have been frozen in dry ice-alcohol mixtures and dried in a thin layer in the frozen state by means of a Cenco pump hooked in series with a trap maintained at  $-40^\circ$  by dry ice and butyl alcohol.

soluble in the absence of salts or in the pH range 4.5 to 7.0. The powders slowly lose their activity over a period of months when stored in a desiccator over  $\text{H}_2\text{SO}_4$  at  $0^\circ$ . Powders prepared by the above methods have quite consistently yielded K fractions active in a unit dose of 0.2 mg. of protein. The Q fractions are less active (unit dose of about 0.3 mg.). On several occasions extracts have been prepared from our K powders by various means (isoelectric precipitation, alumina  $\text{C}\gamma$  adsorption) that have proved to be active in a unit dose as low as 0.077 mg. of protein. The yield as a rule has been small and the preparations quite unstable.

*pH Stability*—In order to determine the optimum pH for storage of extracts the following series of experiments was carried out. Samples of a potent extract adjusted to pH values ranging from 1.5 to 12.3 were stored in stoppered Pyrex flasks at  $0^\circ$  for periods as long as 3 or 4 weeks. The different samples were assayed periodically for their R.Q.-reducing potency. All extracts stored at pH values below 8.0 retained less than 25 per cent of the original activity after 10 days. Extracts stored in the pH range 9.5 to 11.0 were much more stable, while those extracts subjected to pH values above 11.5 lost much of their activity. All solutions, even those stored at pH 3.3 and 12.3, retained small amounts of activity after 3 weeks storage.

Isoelectric precipitation carried out on K fractions in acid solutions (pH 3.3 to 6.5) has often given small amounts of very active material. The total yield, however, even when all fractions are tested, has been very small (losses up to 90 per cent of the initial activity). The factor is much less stable in acid than in basic solution. It does not precipitate sharply at any pH value but tends to come out of solution over a wide pH range, the greater amount precipitating in the neighborhood of pH 6.6 to 5.75. The results have been substantially the same in salt-free electrodialyzed samples. Some of our most potent extracts have been allowed to stand near pH 10.5 for weeks without marked loss of activity. For these reasons we have adopted the practice of storing our extracts made from the K powders in tightly stoppered bottles at pH 9.5 at  $0^\circ$ . They retain their activity well for 1 to 4 weeks. Frozen extracts stored at  $-10^\circ$  are more stable. Repeated freezing and thawing result in marked loss of activity.

*Reduction of Factor by Cysteine*—K powders dissolved in 0.01 M sodium phosphate solution and mixed with a large excess of cysteine (final pH = 8.0) were stored at 0° under nitrogen for a period of 24 hours, after which they were dialyzed against large volumes of 0.01 M sodium carbonate solution (adjusted to pH 9.5 by the addition of HCl) to remove cysteine and cystine. The factor was not inactivated by this treatment. Similar properties have been described for the growth hormone (24).

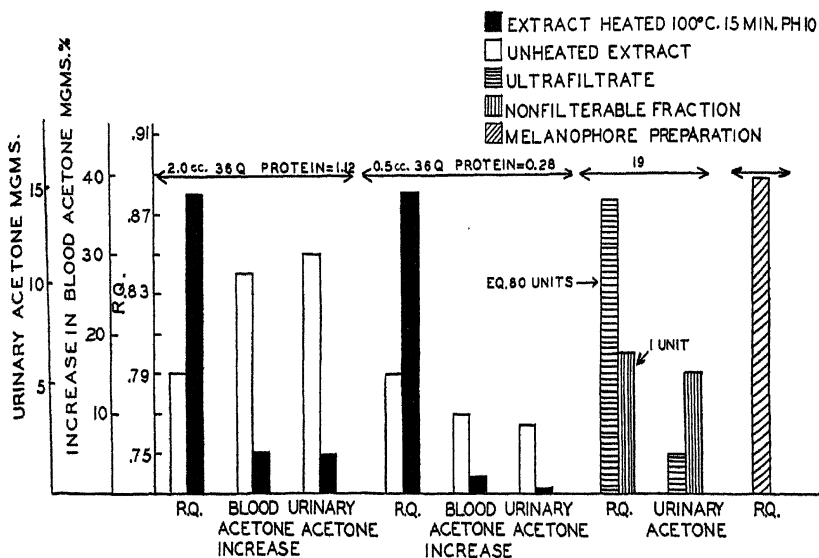


FIG. 4. The effect of heat and ultrafiltration upon the R.Q.-reducing and ketogenic activity of anterior pituitary extract. The ultrafiltration studies were carried out on Extract 19, a crude alkaline extract.

*Heat Stability*—Fig. 4 shows the results of a series of experiments carried out with Fraction 36Q and Extract 19 (a crude alkaline extract). A saline extract of 36Q powder was injected into a series of rats at 2.0 and 0.5 cc. levels. The average of these determinations gave R.Q. values of 0.79. A portion of this material was adjusted to pH 10 and heated to 100° for 15 minutes. Heating resulted in the complete loss of the ability to lower the R.Q. values, and a marked loss of the ketogenic potency as judged

by urinary and blood acetone values. Some workers have obtained comparable results (5, 7, 10); others disagree (16, 18).

*Ultrafiltrability*—A crude alkaline extract of the pituitary was ultrafiltered through a collodion membrane and the ultrafiltrate and non-filtrable portions were tested for their R.Q.-depressing and ketogenic effects (Fig. 4). The ultrafiltrate was injected at a level equivalent to 80 times the unit dose (assuming equal concentrations on the two sides of the membrane) and was found to be inactive. The non-filtrable portion was active in 1/80 the above dose. The ultrafiltrate was tested for urinary ketogenic effects at a dosage level calculated to be 120 times the amount required to give a unit R.Q. response. No significant increase in acetone excretion occurred. The non-filtrable fraction was effective in increasing urinary acetone excretion. The factor is, therefore, not ultrafiltrable, a conclusion which is in agreement with that of some workers (10) but not of others (7, 9, 13, 25).

*Relation to Melanophore Hormone*—A melanophore extract was prepared from the posterior lobe and pars intermedia of beef pituitary glands by the method of Zondek and Krohn (26). This preparation when injected into our test rats did not depress the R.Q. (Fig. 4). The dose administered contained approximately 3000 times the melanophore content found in the unit R.Q.-depressing dose of our anterior lobe preparations. All melanophore preparations were assayed on frogs.

#### *Other Hormones Found in Fraction 36K*

*Thyrotropic Factor*—A solution containing 2.12 mg. of 36K powder was injected subcutaneously into day-old chicks over a 4 day period, according to the method of Bergman and Turner (27). The weights of the thyroid glands were not increased over controls. Hypophysectomized rats injected with a total of 110 R.Q. units over a 10 day period gave subnormal basal metabolic values.

*Adrenocorticotropic Factor*—A solution containing 75 R.Q. units of 36K powder administered intraperitoneally to 21 day-old rats over a 4 day period failed to increase the weight of the adrenals. A total dose of 110 R.Q. units of this powder when administered daily intraperitoneally over a 10 day period to completely hypophysectomized rats (confirmed at autopsy) did not stimulate the

adrenals significantly. The rats, 120 to 140 gm. of body weight at operation, failed to grow or lost weight over a 15 day period before injections were initiated. After 10 days injection, adrenal weights of 10, 14, 16, 14, 12, and 11 mg. were recorded.

*Gonadotropic Factor*—Four daily injections containing 75 R.Q. units of Fraction 36K injected intraperitoneally into 21 day-old rats caused but slight hypertrophy of the gonads. Cysteine-treated samples were inactive when tested at a 110 R.Q. unit level. Hypophysectomized animals (body weight 120 to 140 gm.) treated for 10 days with a total of 110 R.Q. units of Fraction 36K had ovaries weighing 8 to 20 mg.

*Growth Factor*—Adult female rats with stationary body weights injected intraperitoneally daily for 20 days with a total of 110 R.Q. units of Fraction 36K gained on an average of 24 gm. in body weight during the period. After storage for 7 months in a desiccator over  $H_2SO_4$  at  $-3^\circ$ , Fraction 36K was again assayed for growth potency in completely hypophysectomized rats. A total dose of 110 R.Q. units administered over a 10 day period resulted in 17 to 25 gm. of growth (average 23 gm.) during the period. At this time the powder had lost about 60 per cent of its R.Q.-reducing potency. The growth effects had likewise decreased markedly.

*Lactogenic Factor*—The material (Fraction 36K) was tested by both the Lyons (28) and the Riddle *et al.* (29) pigeon methods at dosage levels of 1.5 and 70 R.Q. units intradermally and intramuscularly respectively and caused slight stimulation of the crop glands in each case.

The fraction, therefore, contains small amounts of gonadotropic and lactogenic material and considerable amounts of the growth factor. Extracts treated with cysteine at pH 8.0 according to the method of Meamber, Fraenkel-Conrat, Simpson, and Evans (24) proved to be free of gonadotropic material when tested at a 110 R.Q. unit level but contained small amounts of lactogenic and possibly adrenotropic material.

*Ketogenic Factor*—Many of our different extract preparations have been tested for their ketogenic effects. The following methods have been adopted. Young male rats of body weights of 175 to 250 gm. were fasted 24 hours before urine collection was started. The urine was collected between the 24th and 48th

TABLE I  
*Influence of Anterior Pituitary Extract on Blood and Urinary Acetone Values of Fasted Rat*

Treatment	Group No.	Urinary acetone					Blood acetone			
		No. of determinations	Fore period mean acetone (24th-48th hr.)	Test period mean acetone (48th-72nd hr.)	In-creased excretion of acetone during test period over fore period	No. of animals excreting over 6.0 mg. acetone during test period	No. of determinations	Mean acetone control value (48th hr.)	Mean acetone 4 hrs. after injection (52nd hr.)	In-crease over control level
			mg.	mg.	mg.			mg. per cent	mg. per cent	mg. per cent
Control series										
Rats fasted 24-48 hrs. No treatment	I	129	3.0 0.8-13.8*			5 (Control period)	65	6.5 1.3-27.6*		
Rats fasted 72 hrs. Saline injected 48th hr.	II	7	2.1 1.6-2.9*	4.4 0.3-6.3	2.3	2	7	7.7 6.4-9.6*	8.9 5.2-10.7*	1.2
Rats fasted 72 hrs. 0.2-0.5 unit anterior pituitary extract injected 48th hr.	III	20	3.6 1.4-13.5*	6.6 3.6-17.7	3.0	8	20	9.6 1.3-23.8*	15.4 1.9-31.1	5.8
0.5-1.0 unit anterior pituitary extract injected 48th hr.	IV	7	3.5 1.7-5.5*	6.6 0.7-8.3	3.1	4	7	14.4 5.6-27.6*	19.9 15.8-24.4*	5.5
1.0-2.0 units anterior pituitary extract injected 48th hr.	V	6	2.8 1.3-4.0*	5.6 0.6-6.5	2.8	4	6	11.4 3.9-20.8*	17.1 9.0-24.4	5.7
2.0-3.0 units anterior pituitary extract injected 48th hr.	VI	26	2.9 0.8-6.7*	9.6 3.7-26.1	6.7	15	31	9.1 2.9-17.8*	30.0 7.2-43.2*	20.9

\* Range of values in group.



hours (fore period). Anterior pituitary extracts or saline was then administered by intraperitoneal injection and the urine was collected between the 48th to 72nd hours (test period) and the 72nd to 96th hours (post period). Blood samples were taken at the beginning of the test period and again 4 hours later. Blood acetone bodies were determined by the method of Shipley and Long (10). Total urinary acetone body determinations were carried out by the gravimetric method of Van Slyke with the Denigès reagent (30). A factor of 0.062 was used to convert the

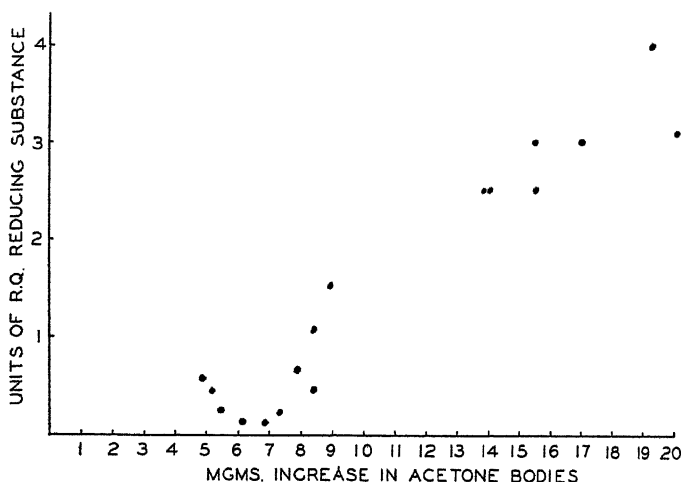


FIG. 5. Summary of the R.Q.-reducing and ketogenic potency of different extracts showing the general parallelism between the two effects. Each point represents an average of five to seven or more separate determinations.

urinary acetone precipitate to acetone. All results are expressed as mg. of acetone.

In general the ketogenic responses of different animals as well as of the same animal on different trials varied widely. This was especially true of the urinary excretion values. The blood ketone levels were somewhat less variable, as has been found by Shipley and Long (10) (see Table I, Group I). After a 48 hour fast the blood acetone levels varied from 1.3 to 27.6 mg. per cent, whereas the urinary values for the fore period (24th to 48th hours of fast) varied from 0.8 to 13.7 mg. per 24 hours.

Table I summarizes the blood and urinary acetone findings for the control (24th to 48th hour of fast) as well as for the test periods (48th to 72nd hours) as affected by the amount of anterior pituitary extract administered. The same extracts were assayed also by the R.Q. depression method. Groups III to V which received increasing amounts of extract showed only a doubtful increase in ketone excretion but had significantly higher blood ketone levels. When 2 to 3 units of anterior pituitary extract were given (Group VI) (a dose which reduced the R.Q. to about 0.78), a significant increase in urinary ketone body excretion as well as a marked rise of blood ketones occurred.

The general parallelism between the R.Q.-reducing effects and the increase in blood acetone body content is shown in Fig. 5. Each point represents an average of 5 to 7 or more individual blood determinations. We have prepared numerous extracts by a wide variety of procedures and have not been able to obtain an extract potent in either the R.Q. effect or the ketogenic effects without being active also in the other.

#### SUMMARY

A method of assay is described for the respiratory quotient-reducing substance of the anterior pituitary gland, based upon the effect of the intraperitoneal injection of the extract upon the respiratory quotient of rats fasted 20 hours before and fed glucose 2 hours after the injection of the extract under assay. An approximate unit is defined as that amount of material which will reduce the respiratory quotient to 0.80 (from the control values of 0.86 to 0.94). A curve has been constructed relating the respiratory quotient to the dosage level. The effects of the same extracts upon the blood and urinary ketone body levels have been studied in the fasted rat.

With these criteria, attempts have been made to concentrate and study the properties of the factor or factors involved. Basic extraction of frozen glands at pH 11.0 has given results far superior to those obtained by neutral, acid, buffer, or saline extraction. The factor is heat-labile ( $100^{\circ}$  at pH 10 for 15 minutes) and non-diffusible through cellophane or collodion membranes. The activity of different extracts does not parallel their melanophore content. The larger part of the factor is salted-out by full

saturation with NaCl or 0.2 to 0.45 saturation with ammonium sulfate. Isoelectrically a large part of the factor precipitates in the region of pH 6.7 to 5.75. The activity of the factor is not destroyed by treatment with excess cysteine at pH 8.0. It is more stable in the pH range 9.5 to 11.0 than at higher or lower pH values. It is much less stable in acid solution. By means of a modified Evans, Cornish, and Simpson alkaline procedure followed by repeated salting-out with 0.2 to 0.45 saturation with  $(\text{NH}_4)_2\text{SO}_4$ , dialysis, and isoelectric precipitation between pH 6.7 and 5.75 fractions have been prepared active in a unit dose of 0.17 mg. of protein. Our best fractions are rich in growth factor and contain only small amounts of lactogenic and adrenocorticotrophic material. The ketogenic and R.Q.-reducing potencies of our fractions run approximately parallel. It has not been possible to separate the two effects.

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# MUCINASE: A BACTERIAL ENZYME WHICH HYDRO- LYZES SYNOVIAL FLUID MUCIN AND OTHER MUCINS\*

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In the course of a series of investigations undertaken in this laboratory to obtain as complete a knowledge as possible of the physiology of normal and pathological joints, investigations concerning the metabolism of synovial fluid mucin were undertaken. The two possible mechanisms for removal of mucin from the joint appear to be enzymatic destruction within the joint or removal by way of the lymphatics. In view of the known difficulty in removing globulin (a smaller molecule than mucin) from the joint (1), it seemed probable that enzymatic activity was the important factor.

In an attempt to obtain evidence of the presence in normal or pathological fluid of an enzyme capable of destroying mucin, specimens of sterile normal and pathological fluids were allowed to stand for varying periods of time at 25° and 37°. In no instance was any decrease in mucin noted nor did any change in mucin occur. However, in the course of other studies on synovial fluid mucin, a specimen of normal synovial fluid was encountered which had lost its viscosity and from which mucin could no longer be precipitated as a ropy mass by the addition of dilute acetic acid. The possibility that the mucin had been destroyed by an enzyme,

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elaborated by contaminating bacteria, was immediately suggested. An anaerobic bacillus, identified as *Clostridium perfringens*,<sup>1</sup> was isolated and found to produce an enzyme which decomposed synovial fluid mucin.

The destruction of mucins or their prosthetic polysaccharides by a bacterial enzyme has been described by other workers. Neuberg and Cahill (2) isolated a bacterial enzyme capable of liberating sulfuric acid and reducing substances from the chondroitin of cartilage. Meyer, Dubos, and Smyth (3) demonstrated that the autolytic enzyme of the pneumococcus hydrolyzed "hyaluronic acid," the polysaccharide obtained from vitreous humor, umbilical cord, and hemolytic streptococci. Since our studies were first undertaken, Meyer, Smyth, and Dawson (4) have reported that the autolytic enzyme of pneumococci hydrolyzes a polysaccharide similar to hyaluronic acid, isolated from synovial fluid.

The enzyme elaborated by *Clostridium perfringens*, which we have designated *mucinase*, hydrolyzes synovial fluid mucin in two stages. The first step produces a decrease in viscosity of the mucin solution and a change in the precipitability of the mucin with acetic acid. In place of the usual ropy, coherent mass, a completely atypical flocculent precipitate is obtained (Fig. 1). This change in precipitability takes place within a few minutes. Neither free amino sugars nor reducing substances are then present. A secondary reaction, proceeding at a much slower rate, liberates amino sugars and reducing substances, which reach a maximum in 24 to 48 hours. All enzyme preparations have shown a similar relation between the rates of the two reactions. No increase of free amino groups is caused by the enzyme.

A polysaccharide, isolated from synovial fluid mucin after tryptic digestion of the protein component, is similarly destroyed by mucinase.

Fluids from many cases of specific infectious and rheumatoid arthritis show a decreased concentration of mucin, a lowered viscosity, and a change in the type of precipitate obtained with

<sup>1</sup> We are indebted to Dr. Louis Dienes and Dr. Helen Arnold of the Massachusetts General Hospital for bacteriological assistance, and to Dr. Ivan Hall of the University of Colorado, Denver, for corroboration of the identity of the organism.

acetic acid. The similarity of these changes to those produced by mucinase (see Fig. 1) suggests that they are caused by enzymatic phenomena.

### 1. Preparation and Purification of Mucinase and Substrate

*Clostridium perfringens* is cultured anaerobically for 18 hours in infusion broth containing a layer of meat. (Composition of broth: meat infusion, 0.5 per cent sodium chloride, 2 per cent Bacto-Peptone.) Omission of the meat results in reduction in the

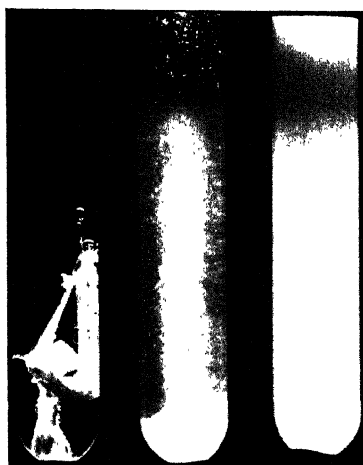


FIG. 1. Change in acetic acid precipitability of mucin during enzymatic digestion. From left to right, typical precipitate of good mucin; precipitate of mucin after partial enzymatic digestion with mucinase; precipitate of mucin after complete breakdown by mucinase.

mucinase activity of the culture. Incubation at 37° is only slightly more favorable than cultivation at 20°. Repeated transfers of the organism into meat-layered infusion broth result in a variation of the characteristics of the culture. This change appears to be irreversible. The broth becomes viscous and it is possible to precipitate a mucoid-like substance with acetone. The concentration of mucinase is rendered difficult by these changes.

500 cc. of an 18 hour broth culture are centrifuged and filtered through a Seitz bacterial filter. (The activity of various broth cultures fluctuates very little.) 1 volume of ice-cold acetone is



added while the temperature is kept below 10°. 0.5 cc. of a saturated calcium chloride solution is added. The calcium phosphate thus formed effectively adsorbs the enzyme. The centrifuged precipitate is washed well and finally suspended in approximately 70 cc. of distilled water. The adsorbent is just dissolved with 3 *N* acetic acid. After centrifuging, the solution is dialyzed against cold distilled water for 48 hours and again centrifuged. The activity is increased approximately 900-fold. There are no detectable amounts of calcium, magnesium, or phosphate ions present. Heating at 60° for 5 minutes destroys its activity. At 4°, the solution is fairly stable, losing about 20 per cent of its activity in 1 month.

Estimation of the activity of mucinase is based on the primary stage of the mucin degradation, as determined by the change in precipitability with dilute acetic acid (Fig. 1). 1 cc. of the enzyme-containing solution is added to 3 cc. of a 0.05 *M* phosphate solution, pH 7.4, containing approximately 15 mg. of mucin and mixed well. The reaction mixture is kept at 25°. Portions are removed at frequent intervals and pipetted into an excess of 1 per cent acetic acid. The end-point is considered reached when mucin fibers no longer appear. A fair accuracy can be obtained after an orienting determination. The complete breakdown of 10 mg. of mucin in 10 minutes corresponds to an activity of 10.

Reducing substances were determined by the method of Hagedorn and Jensen (5). Free amino sugar was determined as glucosamine after Morgan and Elson (6). An Evelyn<sup>4</sup> photoelectric colorimeter with Filter 520 was used.

Viscosity is the relative viscosity at 25°, as measured in a modified Ostwald pipette. The loss in viscosity may be used qualitatively as a test for mucinase action.

For the preparation of synovial fluid mucin, the astragalotibial joints of steers<sup>2</sup> are aspirated within 30 minutes after death. The fluid is centrifuged and diluted with 5 volumes of water. The mucin is precipitated by the addition of acetic acid to a final concentration of 1 per cent. The ropy precipitate is washed with water and dissolved in one-half the original volume of 0.05 *M*

<sup>2</sup> We are indebted to the New England Dressed Meat and Wool Company for their kind cooperation.

secondary sodium phosphate. After solution has taken place (approximately 2 hours), 2 volumes of alcohol are added and mixed well; the precipitation is completed with 1 volume of ether. The precipitate is washed and redissolved in the same volume of 0.05 M secondary sodium phosphate. This solution is poured into 4 volumes of 1 per cent acetic acid; the precipitated mucin is washed and dissolved in 0.5 per cent sodium carbonate. The solution is dialyzed for 2 weeks against cold distilled water, centrifuged sharply, and concentrated by evaporation from the surface of a cellophane sack in the ice box. A 0.2 M solution of phosphate buffer, pH 7.4, is added until the concentration reaches 0.05 M. Upon the addition of dilute acetic acid, this very viscous solution gives the typical white, ropy precipitate, which is insoluble in water. The nitrogen content of the mucin is approximately 12.5 per cent and upon hydrolysis the amino sugar yield is 7 per cent.

The polysaccharide of synovial fluid mucin is obtained from the acetic acid precipitate from 10 liters of synovial fluid which is dissolved in 2 liters of 0.5 per cent sodium carbonate and reprecipitated in 4 volumes of 2 per cent acetic acid. The mucin, redissolved in 2 liters of 0.5 per cent sodium carbonate, is precipitated by the addition of 2 volumes of alcohol acidified with acetic acid, redissolved in 0.5 per cent sodium carbonate, and the pH adjusted to 9. 5 gm. of trypsin (Pfanstiehl 1-110) are added and the mucin allowed to digest 36 hours at 38°. The resulting solution although still viscous gives no precipitate with dilute acetic acid. Trichloroacetic acid is added to a concentration of 10 per cent to precipitate the remaining protein. The solution is then centrifuged, neutralized with potassium hydroxide, and precipitated with 3 volumes of alcohol. The flocculent precipitate is taken up in 600 cc. of water and reprecipitated with 5 volumes of alcohol. The stringy mass is washed well with alcohol acidified with acetic acid, and ether, and dried *in vacuo*. About 3 gm. of the polysaccharide are obtained from 10 liters of fluid. The potassium salt is white and very fluffy. It is sulfur-free but contains traces of phosphorus. It is readily soluble in water, giving clear viscous solutions from which it is not precipitated with dilute acetic acid. A 0.2 per cent solution has a viscosity about 40 times that of water at 25°.

## 2. Characterization of Mucinase

The effect of mucinase on synovial fluid mucin was followed quantitatively to determine whether the action of mucinase is due to two enzyme systems. The rates of reaction of several enzyme preparations were compared for the two stages of the breakdown. The ratio of the rates of reaction was the same for each preparation, which indicates that only one enzyme is responsible for the two reactions.

TABLE I  
*Enzymatic Liberation of Amino Sugars and Reducing Substances  
from Synovial Fluid Mucin*

Preparation	Ac- tivity per cc.	Length of digestion					After acid hy- drolysis
		30 min.	6 hrs.	12 hrs.	25 hrs.	50 hrs.	
Amino sugars as mg. glucosamine per cc.							
Mucinase I.....	75	0.0025	0.01	0.025	0.053	0.062	0.37
“ II.....	160	0.006	0.035	0.067	0.062	0.062	0.37
“ III.....	130	0.004	0.024	0.067	0.062	0.062	0.37
Filtered culture of <i>Clostridium perfrin- gens</i> .....	24	0.004	0.005	0.011	0.022		
Reducing substances as mg. glucose per cc.							
Mucinase I.....	75	0.02	0.12	0.18	0.25	0.29	0.45
“ II.....	160	0.08	0.25	0.30	0.30	0.31	0.45
“ III.....	130	0.05	0.19	0.29	0.30	0.31	0.47
Filtered culture of <i>Clostridium perfrin- gens</i> .....	24	0.12	0.13	0.15	0.20		

1 cc. of each of the mucinase solutions to be tested was added to 20 cc. samples of a buffered mucin solution (5 mg. per cc.) and incubated at 38°. The original viscosity of 33.7 was reduced in each case to approximately 2 after 15 minutes of enzymatic digestion. The data as reproduced in Table I show the similarity of rates for the two stages.

The action of mucinase on the polysaccharide from synovial fluid mucin was studied in similar experiments. The original

viscosity of 59.6 was reduced to 1.5 after 15 minutes of enzymatic digestion. Amino sugars and reducing substances were liberated as shown below.

	30 min.	24 hrs.	48 hrs.	After acid hydrolysis
Amino sugars as mg. glucosamine per cc.....	0.0025	0.03	0.06	0.282
Reducing substances as mg. glucose per cc.....	0.012	0.131	0.189	0.385

The activity of the mucinase preparation was 130 per cc.

The specificity of mucinase towards other mucoids than that obtained from synovial fluid was determined qualitatively. Thus, if a buffered viscous solution of the mucoid lost its viscosity and characteristic precipitability after incubation with mucinase, it was considered a possible substrate for the enzyme.

Mucinase did not affect gastric mucin (human or swine) or salivary mucin (human), both of which are secreted by mucous membranes, or chondroitinsulfuric acid obtained from cartilage. Cattle cartilage suspended for 1 week in an enzyme solution showed neither gross nor microscopic changes.

Viscous solutions of mucins obtained from umbilical cord (human), and loose abdominal connective tissue fascia (rabbit) by extraction of these tissues with secondary sodium phosphate, and the clear viscous solution of macerated vitreous bodies (cattle) all gave typical mucin precipitates. In each case the viscosity and characteristic precipitability were rapidly destroyed by mucinase.

The control experiments, without added mucinase, were negative except in the case of vitreous mucin. Vitreous mucin was destroyed after standing several hours at room temperature. Furthermore, centrifuged vitreous humor caused the breakdown of 10 mg. of added synovial fluid mucin during overnight digestion. A substance with mucinase activity is apparently present in the eye in small amounts. Meyer *et al.* (4) report an enzyme similar to the autolytic enzyme of pneumococcus in the ciliary body. In the vitreous humor the enzymatic activity is apparently due to ascorbic acid (7).

Further characterization of the enzyme is given by the following experiments which show the effect of various conditions on the rate of reaction of mucinase.

### *Effect of Substrate Concentration*

1 cc. of a mucinase solution was added to varying amounts of mucin dissolved in buffer. The breakdown times are recorded.

Mucin, mg.....	10	20	30	40	60	100
Time, min.....	4	8	11½	15½	23	41

The rate of reaction is constant and independent of the amount of mucin present. The accumulation of the primary degradation product does not affect the rate of mucinase reaction.

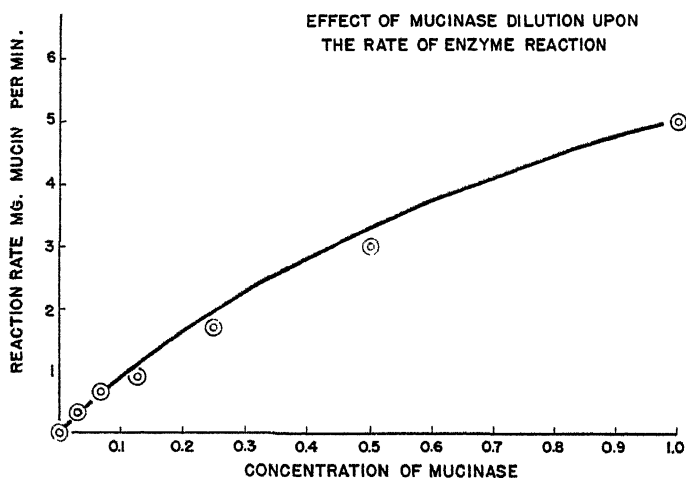


FIG. 2

### *Effect of Enzyme Concentration*

Several dilutions of a purified mucinase solution were made. 1 cc. of each enzyme solution was added to 3 cc. of the mucin solution containing 15 mg. Fig. 2 shows the rate of enzyme reaction plotted against an arbitrary enzyme concentration. The curve shown is typical of three obtained with different enzyme preparations. The rate of reaction is not directly proportional

to the amount of enzyme present, the more dilute solutions being more active. This effect may be due to protective colloids.

### *Effect of Salt Concentration*

Preliminary experiments indicated that dialyzed mucinase preparations did not cause a breakdown of dialyzed mucin. The

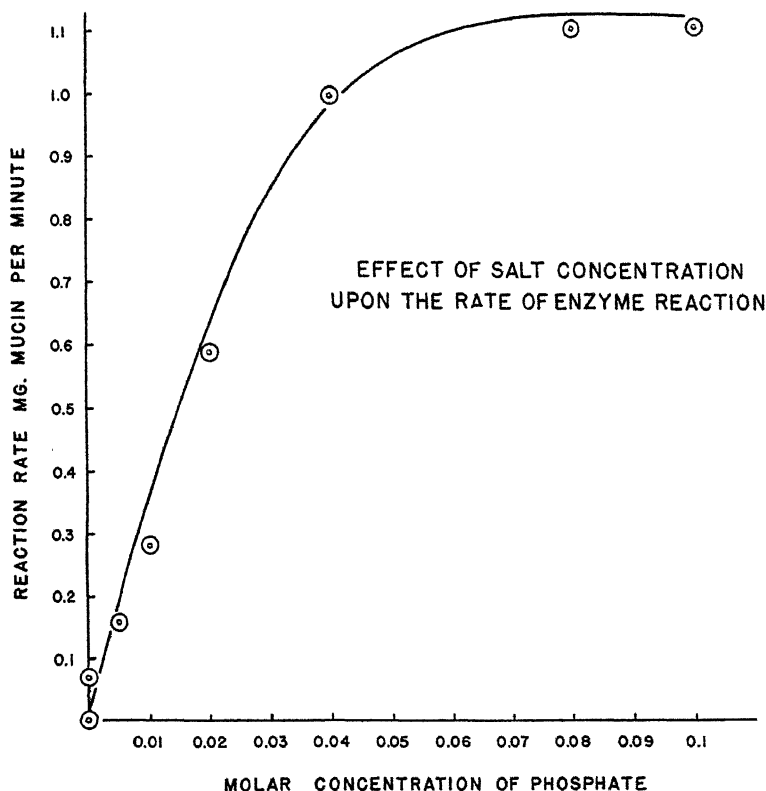


Fig. 3

addition of small amounts of salt activated the reaction. The effect of salt concentration was further investigated. 0.5 cc. of mucinase was added to 2 cc. of a dialyzed mucin solution containing 10 mg. of mucin and 1 cc. of phosphate buffer, pH 7.4, of varying concentration.

Fig. 3 shows the increase in the reaction rate of mucinase with

increasing buffer concentration. Citrate, chloride, and sulfate salts reactivate the enzyme similarly.

### *Effect of Viscosity*

1 cc. of a purified mucinase solution was added to 10 mg. of mucin dissolved respectively in 2, 4, 6, 8, and 16 cc. of 0.05 M phosphate buffer. The viscosities of these solutions ranged from 24 to 2 respectively. The change in characteristic precipitability took place in each case in 5 minutes. Substrate dilution with resultant lowering of viscosity does not affect the rate of enzyme reaction.

### *Effect of Temperature*

The breakdown of 10 mg. of mucin was carried out in a water bath kept at the desired temperature. The time in minutes required for breakdown of the mucin in two experiments is recorded.

Temperature, °C.....	0	10]	20	30	40	50
Experiment I.....	42	24	13	7½	4	2¼
“ II.....	31	18	10	6½	3½	2
$K_{10}$ .....	1.7	1.8	1.7	1.8	1.8	

The average  $K_{10}$  is 1.75. Similar values have been reported for other enzyme reactions. Above 50°, destruction of the enzyme takes place rapidly.

### *Effect of pH*

5 cc. of 0.5 N sodium chloride containing different amounts of 0.5 N hydrochloric acid or of 0.5 N sodium hydroxide to adjust the pH were added to 10 cc. of a dialyzed mucin solution. The pH was determined with the glass electrode. The reaction times for the breakdown of the mucin in 3 cc. of these solutions by 1 cc. of a dialyzed mucinase solution were measured.

pH .....	2.5	3.9	4.3	5.3	5.7	6.4	6.8	7.4	8.5	9.1
Time, min.....		8½	9	9½	8½	8½	9	10½	14	

The active optimal range is broad, extending from pH 3.9 to 8.5. In the alkaline range, the activity diminishes until at pH 9.1

complete inactivity has taken place. Because of precipitation of the mucin, the enzyme activity could not be measured between pH 3.9 and 2.5.

### *Inhibiting Effect of Certain Substances*

1 cc. of a solution of substance to be tested was added to 1 cc. of a mucinase solution and the mixture added to 3 cc. of a 0.05 M phosphate buffer solution containing 15 mg. of mucin. The final concentration of the inhibitor and the time of reaction are recorded:

Water control.....	5 min.
Potassium cyanide, 0.03 M .....	60 "
Sodium arsenite, 0.03 " .....	60 "
Iodine, 0.02 " .....	No breakdown
" + thiosulfate .....	" "
" + sodium arsenite .....	" "
" + " sulfite .....	" "

Caprylic alcohol, iodoacetic acid, maleic acid, hydrosulfite, bisulfite, sulfite, thiosulfate, fluoride, hydrogen peroxide, phenol, heparin, urethane, cysteine, cystine, ascorbic acid, as well as 2,6-dichlorophenol indophenol, methylene blue, leuco neutral red, and neutral red in concentrations up to 2 per cent did not inhibit the mucinase.

The inhibiting effect of several of the above poisons on the liberation of amino sugars and reducing substances was studied (Table II). The specificity of action is the same for both stages of the enzymatic mucin degradation.

1 cc. of a mucinase solution was added to 50 cc. of a mucin solution containing 250 mg. of mucin. After 30 minutes, the loss in viscosity was complete. 5 cc. of the solution were added to the tubes containing the inhibitor and the tubes filled to 7 cc. Increases in reducing substances and amino sugars were determined.

We have not been able to reverse the action of iodine. This observation coupled with the inactivation by mild reducing substances, such as potassium cyanide and sodium arsenite, as well as the ineffectiveness of maleic acid, a specific inhibitor for enzymes with an active sulfide group (8), precludes the possibility that such a sulfide linkage is responsible for the mucinase activity. Stronger oxidizing and reducing substances are among



TABLE II

*Effect of Inhibitors on Enzymatic Hydrolysis of Synovial Fluid Mucin*

Inhibitor	0 hr.	18 hrs.	42 hrs.
Reducing substances as mg. glucose per cc.			
0.03 M potassium cyanide.....	0.01	0.02	0.02
0.02 " sodium arsenite.....			
0.1 " " oxalate.....	0.01	0.12	0.31
0.1 " " sulfite.....	0.03	0.14	0.28
0.02 " iodine .....	0.01	0.02	0.02
0.02 " " + thiosulfate.....	0.24	0.25	0.25
Control.....	0.00	0.18	0.38
Amino sugars as mg. glucosamine per cc.			
0.03 M potassium cyanide.....	0.003	0.002	0.003
0.02 " sodium arsenite.....	0.002	0.002	0.004
0.1 " " oxalate.....	0.003	0.0125	0.02
0.1 " " sulfite.....	0.003	0.0125	0.018
0.02 " iodine .....	0.002	0.002	0.002
0.02 " " + thiosulfate.....	0.003	0.002	0.002
Control.....	0.002	0.02	0.03

TABLE III

*Mucinasase Activity of Bacterial Cultures*

<i>Clostridium perfringens</i> .....	100	<i>Bacterium proteus</i> .....	0
<i>Streptococcus bovis</i> .....	30	" <i>prodigiosum</i> .....	0
<i>Pneumococcus</i> Group VIII... 15		" <i>pyocyaneum</i> .....	0
" " XIII... 12		<i>Clostridium tetani</i> .....	0
<i>Clostridium oedematiens</i> ..... 1-2		" <i>botulinum</i> .....	0
<i>Vibrio septique</i> ..... 3-4		<i>Gonococcus</i> .....	0
<i>Streptococcus viridans</i> , 1 of 4 cultures..... 25		<i>Staphylococcus aureus</i> and <i>albus</i> ... 0	
<i>Streptococcus hemolyticus</i> , 1 of 8 cultures..... 15		<i>Streptococcus hemolyticus scarlatinae</i> ..... 0	
<i>Bacterium coli</i> ..... 0		<i>Diphtheroid</i> ..... 0	
<i>Bacillus subtilis</i> ..... 0		<i>Bacterium dysenteriae</i> , Sonn�..... 0	
		<i>Clostridium bifermentans</i> ..... 0	

the substances which do not inhibit. It is, therefore, probable that oxidation or reduction processes play no important r le in the activation of mucinasase.

### 3. *Distribution of Mucinae*

Cultures of various organisms were studied in an attempt to determine the distribution of the enzyme and a possible correlation between mucinase activity and the association of organisms with various joint diseases. Cultures of organisms known to produce septic joints with subsequent destruction of mucin (such as  $\beta$ -hemolytic streptococcus, pneumococcus, gonococcus, and *Bacterium coli*) were found to have little or no mucinase activity. Table III shows the relative activities of broth cultures of various organisms. The cultures used were 24 hour broth cultures of type strains. It is possible that mucinase activity could be increased or even induced by repeated transplantations in broth containing mucin or its polysaccharide.

### DISCUSSION

The degradation of synovial fluid mucin or its polysaccharide, as catalyzed by the bacterial enzyme, mucinase, takes place in two distinct steps. The first step results in a loss of viscosity and change in precipitability with acetic acid. This is probably a depolymerization or disaggregation. The second stage liberates free amino sugars and reducing substances and is apparently a hydrolysis. Complete hydrolysis, however, is not produced, for only 20 per cent of the total amino sugars and 70 per cent of the total reducing substances, as determined after acid hydrolysis, are liberated. Isolation and identification of the end-products will be necessary to describe adequately the action of the enzyme.

Whether or not mucinase is a single enzyme catalyzing both stages of the destruction of mucin is not certain. The fact that all enzyme preparations examined had the same relation of reaction times of the two stages suggests that it is only one enzyme. The similar specificity of inhibitors also indicates a single enzyme. However, comparison of the destruction of mucin to the amylolysis of starch suggests the possible presence of two enzymes. The rapid loss of viscosity, the subsequent slower liberation of reducing substances, and the reversible inactivation of the mucinase reaction by dialysis are similar to amylolysis. Amylase preparations from malt or pancreas have no effect on synovial fluid mucin. However, mucinase causes a loss of viscosity of starch paste. The amylase causing the loss of viscosity has been shown to be distinct

from that causing the liberation of reducing substances (9). Further attempts should therefore be made to separate the two activities of mucinase.

The liberation of reducing substances from synovial fluid mucin, as catalyzed by mucinase preparations, is similar to the action of the autolytic enzyme of pneumococcus on hyaluronic acid. However, the characteristics of the two enzymes differ in several respects (Table IV).

The attempt to demonstrate the presence of mucinase in the joint effusions of patients with rheumatoid or septic arthritis or in cultures of organisms known to be associated with joint diseases has not been successful. This does not, however, exclude the

TABLE IV  
*Comparison of Mucinase and "Autolytic Enzyme of Pneumococcus"*

	Mucinase	Autolytic enzyme of pneumococcus (3, 4)
Lysis of pneumococci.....	—	+
Effect of alcohol or acetone.....	None	Inactivates
“ “ iodine.....	Irreversibly inactivates	Reversibly inactivates
“ “ arsenite.....	Inactivates	Reactivates
“ “ cyanide.....	“	None
“ “ hydrogen peroxide.....	None	Inactivates
pH range.....	3.9-8.5	4.5-8.0

possibility that an enzyme causing a similar depolymerization is responsible for the degradation of mucin in pathological joints. It is interesting that Meyer *et al.* (4) have been able to isolate from the hemolytic streptococcus an enzyme which liberates reducing sugars from hyaluronic acid. This enzyme is endogenous. We, however, have not been able to show mucinase activity even in those joint fluids heavily infected with virulent hemolytic streptococci.

The results of other experiments on the modes of breakdown of mucin suggest two other mechanisms, in addition to the possible presence of an enzyme similar to mucinase, which may take part in the metabolism of mucin both in normal and in pathological joints. The first stage of the breakdown of mucin is catalyzed

rapidly by ascorbic acid-peroxide (7) and very slowly by serum phosphatase.<sup>3</sup> It is possible that one of these systems is responsible for the catabolism of mucin.

The specificity of mucinase is significant. It does not hydrolyze mucins from mucous membranes and glands, but does hydrolyze mucins formed by mesothelial tissues (umbilical, synovial, and connective tissue) as well as vitreous mucin, the histogenetic origin of which is unknown. This specificity suggests that the mucin extracted from connective tissue is identical with that of synovial fluid and gives further support for the concept that synovial fluid is formed, at least in part, by the connective tissue cells surrounding the joints (10). The fact that the mucins of the tissues so regularly involved in rheumatoid arthritis are digested by mucinase suggests that the presence of mucin in these tissues is of fundamental importance in the localization of this disease.

#### SUMMARY

1. An enzyme (or enzymes), *mucinase*, may be isolated from broth cultures of *Clostridium perfringens*.

Mucinase can be purified and concentrated 900-fold by adsorption on calcium phosphate from a 50 per cent acetone solution.

Mucinase causes loss of viscosity of solutions of synovial fluid mucin or of the prosthetic polysaccharide, and subsequent liberation of amino sugars and reducing substances.

Mucinase is active in the pH range 3.9 to 8.5. The temperature coefficient is  $K_{10}$  1.75. Inactivation takes place at 60°. Removal of salts causes inactivation which is reversible. Mucinase is inactivated irreversibly by cyanide, arsenite, or iodine.

2. Mucinase is not peculiar to *Clostridium perfringens* but is present in varying concentrations in the broth cultures of several other microorganisms.

3. Mucinase hydrolyzes also the mucins of vitreous humor, umbilical cord, and connective tissue, but does not hydrolyze the mucins from mucous membranes and glands.

<sup>3</sup> We are indebted to Dr. Thannhauser for the serum phosphatase which was a concentrated serum obtained from a dog with a ligated common bile duct.

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# THE CONSTITUTION OF CONJUGATED PHENOL-PHTHALEIN FORMED IN THE ANIMAL BODY

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The physiological fate of phenolphthalein in the organism, which is dealt with here, has been studied since the discovery by Vámosy (1) in 1901 of its action as a cathartic. In 1909, Fleig (2) remarked that, since there was an increased secretion of ethereal sulfates in the urine when phenolphthalein was given, the substance is probably eliminated as a *sulfo-conjugué*. In dogs a small part, he believed, seemed to be excreted as a *glycuro-conjugué*, but no evidence was presented. In 1909, while looking for a cathartic which was soluble and which could be injected, Abel and Rowntree (3) found conjugated phenolphthalein and conjugated phenol-tetrachlorophthalein in the bile and urine after injection of the simple substances into dogs.

Later Fantus and Dyniewicz (4) were able to detect phenolphthalein in conjugated form in both the blood stream and the urine after administration of the compound. With human beings they found only conjugated phenolphthalein in the urine when the doses taken were small (30 to 60 mg.), while with larger doses (300 mg.) free phenolphthalein was present in addition to the conjugate. In the same laboratory, blood serum taken at a time when conjugated phenolphthalein was present was used by Rosenthal (5) in a study on the sensitization of animals to the substance.

In the course of experiments on the sensitization of animals to phenolphthalein the present writer was led to the investigation of the nature, as yet not established, of the compound occurring in the serum and urine of animals treated with phenolphthalein.

## EXPERIMENTAL

Rabbits and guinea pigs were treated as in the experiment of Rosenthal (5) by injecting colloidal phenolphthalein-intramuscu-



larly or intracutaneously. Rabbits were injected with 25 ml., guinea pigs with 10 ml., of a 2 per cent solution prepared by the method of Fantus and Dyniewicz (6). 5 gm. of gelatin were dissolved in 50 ml. of hot water, autoclaved, and combined with a solution of 2 gm. of phenolphthalein in 50 ml. of 0.4 N sodium

TABLE I

*Excretion of Phenolphthalein in Urine of Rabbits*

Each rabbit received, by intramuscular or intracutaneous injection, 25 ml. of a colloidal solution, containing 500 mg. of phenolphthalein.

Days after injection	Rabbit I. Intramuscular			Rabbit II. Intramuscular		
	Urine	Free phenolphthalein	Combined phenolphthalein*	Urine	Free phenolphthalein	Combined phenolphthalein*
	ml.	mg.	mg.	ml.	mg.	mg.
1	0			0		
2	0			0		
3	140	0.1	16	97	0.2	8
4	160	0	3	0		
5	100	0	2	65	0	3
6	113	0	0.4	130	0	1
Total recovered...		0.1	21.4		0.2	12

	Rabbit III. Intracutaneous			Rabbit IV. Intracutaneous		
1	0			7	0	0.1
2	103	0.01	91	81	0.7	220
3	0			0		
4	133	0	6	25	2	5
5	80	0	5	82	0	4
6	190	0	8	40	0	0.3
7	40	0	1			
Total recovered...		0.01	111		2.7	229.4

\* Combined phenolphthalein in terms of its phenolphthalein content.

hydroxide previously autoclaved. After mixing, the sodium hydroxide was neutralized by passing carbon dioxide over the surface until the color was just discharged. After injection by one of the two routes, each animal was placed in a metabolism cage and the dark colored urine was collected in bottles containing

toluene for 3 or 4 days. Table I shows the daily rate of excretion in such an experiment.

It can be seen from Table I that at best only about 45 per cent of the phenolphthalein administered was recovered, indicating that a large proportion had been broken down.

In the determination of free phenolphthalein, the urine was freed of protein by precipitation with acetone, which was then removed *in vacuo*. The resulting urine was extracted with ether until the last extract gave no evidence of phenolphthalein. The combined extracts were filtered and the filtrate evaporated to dryness. The residue was taken up in a glycine buffer of pH 10.2 and the color of the resulting solution was compared with known phenolphthalein solutions.

After ether extraction of free phenolphthalein, the urine was acidified to Congo red with hydrochloric acid and shaken with several portions of ethyl acetate in order to extract combined phenolphthalein. Three or four extractions were required to remove all the conjugate from the solution. The ethyl acetate extracts were pooled and filtered, and to estimate the phenolphthalein present an aliquot portion was hydrolyzed with 2 N hydrochloric acid at 100° for 30 minutes to 1 hour. The color developed at pH 10.2 was compared with standards as above. The ethyl acetate extraction served to eliminate the highly colored constituents of the urine which would have interfered in the color comparison.

The feces showed traces of free, but no conjugated phenolphthalein.

In order to secure the phenolphthalein conjugate formed in the animal body, the urine of treated animals was freed of contaminants, such as small quantities of feces, filtered, and stored in the ice chest under toluene until the quantity desired for processing at one time had been collected. It was then treated with 3 volumes of acetone to remove protein, the flocculent precipitate being allowed to settle, and then filtered off. The acetone was evaporated from the filtrate under reduced pressure, the resulting fluid being dark brown. This was extracted with ether several times in order to remove free phenolphthalein. At the beginning of the work the solution at this point was precipitated with lead acetate and the precipitate was eluted with neutral phosphate (pH

6 to 7); it was later found possible to omit this step. The fluid was acidified to Congo red with hydrochloric acid and extracted several times with ethyl acetate. The pale yellow extract was filtered, and then decolorized with charcoal.

Since the substance was soluble in organic solvents only when in a strongly acid condition, it appeared to be acidic in nature. A strongly positive result with the naphthoresorcinol test indicated the presence of a uronic acid.

A number of unsuccessful attempts were made to obtain a crystalline derivative of the compound, several alkaloids and metallic salts being used. A crystalline salt was finally obtained by the use of cinchonidine, an alkaloid whose successful use in the separation and crystallization of a uronic acid derivative had been reported by Heidelberger and Kendall (7).

The decolorized solution of the phenolphthalein conjugate in ethyl acetate was mixed with an excess of cinchonidine dissolved in ethyl acetate. An amorphous precipitate appeared which began to crystallize after standing for a short time. The crystallization was completed by allowing the mixture to stand overnight at room temperature. The crystalline product was filtered off and washed with ethyl acetate.

Purification of the product was made possible by the fact that in boiling dioxane a part of the material went into solution easily, while the rest was difficultly soluble. In this manner two fractions were obtained, an undissolved white residue and a pale yellow filtrate, which on cooling deposited minute prisms. These crystals seemed to be a mixture, since after being extracted with boiling water the residue yielded, on crystallization from dioxane, a substance resembling in all respects the cinchonidine compound described below.

The relatively insoluble white residue was brought into solution by boiling with a large volume of dioxane. After six or seven recrystallizations from this solvent, the substance separated in long white needles grouped in sheaves. Further recrystallizations from absolute alcohol yielded very long, fine white needles. When heated rapidly, the crystals decomposed at about 190° without melting.

When alkali was added to a water suspension of the crystals, no pink color was seen, but the solution became faintly yellow. How-

ever, when the material was heated with 2 N hydrochloric acid at 100° for 30 minutes to 1 hour and then made alkaline, the typical phenolphthalein color was developed. When this pink solution was just decolorized with acid, and extracted with ether, a substance was obtained from the ether extract which crystallized from 50 per cent alcohol, and had a melting point of 258.0–258.5°. A mixed melting point with phenolphthalein gave no depression.

A naphthoresorcinol test on a water suspension of the crystalline cinchonidine compound gave the ether-soluble purple color typical of uronic acids. This also gave an absorption in the yellow as with uronic acids.

In order to obtain the phenolphthalein conjugate as a free acid, an acidified water suspension of the cinchonidine compound was extracted with ethyl acetate. The extract was filtered and evaporated to dryness, giving a colorless gum. This was readily soluble in cold methanol, ethanol, ethyl acetate, dioxane, and pyridine; slightly soluble in ether; insoluble in benzene or toluene. It was slightly soluble in cold, and soluble in hot water, separating on cooling as a colorless viscous mass. The water solution was distinctly acid to litmus.

The conjugated phenolphthalein is hydrolyzed by acids, but resists boiling for 20 minutes in 4 N sodium hydroxide. Fehling's solution and Tollens' reagent are not reduced by the conjugate. If the solution is first hydrolyzed with dilute acid, both Fehling's solution and Tollens' reagent are reduced in the cold. These two simple tests indicate that the linkage between phenolphthalein and the uronic acid is split by acid and involves the aldehyde group, which seems to point to a glycoside linkage. This is further confirmed by the fact that mutarotation was not observed.

As a further test for this type of binding, enzyme hydrolysis was employed. Helferich (8) found that almond emulsin splits those  $\beta$ -glycosides whose sugar has the same configuration on the first 3 carbons as has  $\beta$ -glucose. This does not, however, seem to apply to  $\beta$ -glucuronides, since Bergmann (9) could not hydrolyze  $\beta$ -naphthol- $\beta$ -glucuronide with emulsin. In fact, Helferich and Sparmberg (10) stated that even though there was some evidence of splitting by this enzyme of *l*-menthol- $\beta$ -glucuronide, the action was not like that on the  $\beta$ -glucosides. They suggested that some special enzyme is required to hydrolyze  $\beta$ -glucuronides. This

enzyme Masamune (11) found in an extract of ground kidney, and called  $\beta$ -glucuronidase.

With crude almond emulsin, traces of free phenolphthalein were liberated from the conjugate after 48 hours. On the other hand, the hydrolytic action of crude kidney extract was noticed in less than 30 minutes and was very strong after 3 hours. These experiments indicate that the linkage in conjugated phenolphthalein is of the  $\beta$ -glucuronide type.

In alkaline solution the conjugate has a yellow color. Early work on phenolphthalein derivatives showed that its monomethyl ether, when treated with an alkali, gives a yellow solution in contrast to its dimethyl ether (12, 13). This and the above qualitative tests would appear to show the crystalline compound to be the cinchonidine salt of phenolphthalein-mono- $\beta$ -glucuronide.

The cinchonidine compound, crystallized from alcohol, was analyzed (a) after being dried in a vacuum at 80°; almost the same values (b) were obtained after recrystallization and drying at 80° in a high vacuum. The figures obtained correspond very closely to those required by a monoalcoholate.

(a) 4.402 mg. substance:	10.900 mg. CO <sub>2</sub> and 2.350 mg. H <sub>2</sub> O
(b) 4.830 " "	: 11.960 " " " 2.620 " "
(c) 9.515 " "	: 0.297 ml. N <sub>2</sub> (761 mm. at 27°)
C <sub>45</sub> H <sub>44</sub> O <sub>11</sub> N <sub>2</sub> .	Calculated. C 68.49, H 5.6, N 3.55
C <sub>45</sub> H <sub>44</sub> O <sub>11</sub> N <sub>2</sub> ·C <sub>2</sub> H <sub>5</sub> OH.	" " 67.59, " 6.0, " 3.36
	Found. (a) " 67.53, (a) " 6.0, (c) " 3.55
	" (b) " 67.53, (b) " 6.1

The alcoholate crystals were again crystallized from dioxane, somewhat prolonged boiling being required to dissolve them completely. After the solution had stood overnight, a crop of crystals was obtained which differed from those of the alcoholate in that they were much more easily soluble in boiling dioxane. After recrystallization from dioxane, values corresponding to the cinchonidine salt, combined with 1 mole of dioxane, were obtained.<sup>1</sup>

4.192 mg. substance:	10.270 mg. CO <sub>2</sub> and 2.330 mg. H <sub>2</sub> O
C <sub>45</sub> H <sub>44</sub> O <sub>11</sub> N <sub>2</sub> .	Calculated. C 68.49, H 5.6
C <sub>45</sub> H <sub>44</sub> O <sub>11</sub> N <sub>2</sub> ·C <sub>4</sub> H <sub>8</sub> O <sub>2</sub> .	" " 67.09, " 6.0
	Found. " 66.80, " 6.2

<sup>1</sup> The Pregl determinations for this paper were kindly performed by Dr. Elek in the laboratory of Dr. P. A. Levene.

The phenolphthalein content of the molecule was determined colorimetrically with the Zeiss Pulfrich photometer. A weighed sample of the alcoholate was hydrolyzed with 4 N hydrochloric acid at 100° for 1 hour. This treatment was found sufficient to insure complete hydrolysis. The resulting solution was almost neutralized and extracted with several portions of ether. The ether extracts were combined, evaporated to dryness, and the residue transferred to a volumetric flask with enough alcohol to make 20 per cent of the final volume. 2 ml. of this solution were mixed with 2 ml. of a glycine buffer of pH 10.2 (14). Comparison in the photometer with standard solutions of phenolphthalein similarly prepared in 20 per cent alcohol solution gave the following result.

$C_{45}H_{44}O_{11}N_2 \cdot C_2H_5OH$ .	Calculated.	Phenolphthalein,	38.1%
	Found.	"	39%

A solution of the alcoholate was made in 80 per cent alcohol, in which it is more soluble than in absolute alcohol, by dissolving 98.7 mg. of the crystals in 5 ml. of solution. The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-2.54^\circ \times 100}{2 \times 1.97} = -64.5^\circ \text{ (in 80\% alcohol)}$$

The rotation remained constant over a period of 24 hours.

The blood sera of treated rabbits and guinea pigs also contained a substance which gave the qualitative tests for the phenolphthalein-monoglucuronide.

The experiments described show that phenolphthalein injected into rabbits and guinea pigs is eliminated as a glucuronide. No phenolphthalein sulfate was found in the ethyl acetate extract described above, which contained all the bound phenolphthalein. There was organically bound sulfate in the urine, *i.e.* giving a barium precipitate only after hydrolysis with hydrochloric acid, but this contained no phenolphthalein. Also present in this fluid was a considerable quantity of uronic acid, not combined to phenolphthalein, detectable by the naphthoresorcinol test.

#### SUMMARY

Phenolphthalein injected into rabbits and guinea pigs is conjugated with glucuronic acid. The conjugate was isolated from

the urine and crystallized as the cinchonidine salt, with 1 molecule of alcohol or dioxane of crystallization. The properties of the conjugate are described and it is shown to be phenolphthalein-mono- $\beta$ -glucuronide.

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## LETTERS TO THE EDITORS

### THE VITAMIN K ACTIVITY OF 4-AMINO-2-METHYL-1-NAPHTHOL AND 4-AMINO-3-METHYL-1-NAPHTHOL

*Sirs:*

Considerable interest has been manifested in potent water-soluble vitamin K preparations. Of the various compounds prepared in this laboratory, we find that 4-amino-2-methyl-1-naphthol and 4-amino-3-methyl-1-naphthol are the most promising. As the hydrochlorides they are readily soluble in water or saline solution and can be given either orally or parenterally.

	Sample No.	Dose	No. of chicks	Per cent clotting under 10 min.	Average clotting time	Chick units per mg.	Potency based on 2-methyl-1,4-naphthoquinone
		$\gamma$			min.		units per mg.
4-Amino-2-methyl-1-naphthol*	I	0.30	12	58	10.7	2090	1045
		0.45	12	83	7.0		
	II	0.35	12	33	16.2	2468	1234
		0.70	12	73	13.8		
	III	0.40	12	42	11.4	2740	1370
		0.65	12	67	8.6		
	IV	0.40	10	30	18.0	2450	1225
		0.75	10	80	6.1		
	V	0.40	15	29	18.1	2323	1162
		0.60	15	50	12.8		
Average (5 tests).....						2415	1208
4-Amino-3-methyl-1-naphthol*		0.9	15	53	12.6	1567	784
		1.2	15	67	9.3		
		1.8	15	79	6.2		

\* Both compounds were administered orally in the form of their water-soluble hydrochlorides.

The potency of the two preparations is given in the accompanying table expressed as curative chick units and also in terms of the



standard 2-methyl-1,4-naphthoquinone. 4-Amino-2-methyl-1-naphthol is almost 3 times as potent as vitamin K<sub>1</sub>.<sup>1</sup> Clinically, the former has been found to give excellent results. Daily doses ranging from 3600 to 7200 curative chick units given intravenously restored to normal severe prothrombin deficiency of obstructive jaundice within 2 to 3 days. Infants have responded within 12 hours to a dosage of 1800 units, as have dogs with severe prothrombin depletion induced by biliary fistula.

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<sup>1</sup> Emmett, A. D., Brown, R. A., and Kamm, O., *J. Biol. Chem.*, **132**, 467 (1940).

## ON HYDROXYLYSINE

*Sirs:*

*Structure*—The following evidence, added to that previously published,<sup>1</sup> indicates with some degree of probability that the diamino acid crystallized from gelatin<sup>1</sup> has one of the two following structures,  $\text{CH}_2(\text{NH}_2) \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$  or  $\text{CH}_2(\text{OH}) \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ .

Electrometric microtitration (by Ha. and K.) shows three buffer groups with  $\text{pK}'$  values of 2.20, 8.70, and 9.50. Lysine under the same conditions gave  $\text{pK}'$  values of 2.20, 8.90, and 10.28. The difference of 0.8 between the  $\text{pK}'$  values of the most alkaline buffer groups of the respective amino acids indicates that the hydroxyl group of the hydroxylysine is probably on the carbon adjacent to that bearing the most basic  $\text{NH}_2$  group, which from comparisons with other amino acids is presumably the non- $\alpha$   $\text{NH}_2$ .

If hydroxylysine is treated with an excess of periodate at pH 8 to 12, there is an instantaneous consumption of 1 atom of oxygen from the periodate, with formation of 1 mole each of ammonia and of formaldehyde, which was isolated as dimedon compound.<sup>2</sup> According to Malaprade<sup>3</sup> and Criegee<sup>4</sup> it is characteristic of periodic acid to split a chain between 2 carbon atoms bearing respectively OH and OH or OH and  $\text{NH}_2$  groups, and Nicolet and Shinn<sup>5</sup> have recently confirmed this rule with the hydroxyamino acids, threonine and serine. Our results with periodate therefore agree with the titration curves in locating the OH and one  $\text{NH}_2$  on adjacent carbons. The formaldehyde formation shows that one of these carbons is at the end of the chain; the  $\text{NH}_2$  of the  $-\text{CH}(\text{OH})-\text{CH}(\text{NH}_2)-$  group must accordingly be on either the terminal or the next carbon.

<sup>1</sup> Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyen, D. A., *Proc. Soc. Exp. Biol. and Med.*, **38**, 548 (1938).

<sup>2</sup> Vorländer, D., *Z. anal. Chem.*, **77**, 241 (1929).

<sup>3</sup> Malaprade, L., *Bull. Soc. chim.*, **1**, 833 (1934).

<sup>4</sup> Criegee, R., *Angew. Chem.*, **50**, 153 (1937).

<sup>5</sup> Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939).

Neither the titration curve nor the composition of hydroxylysine preparations from acid solutions gives evidence of lactone formation. The absence of lactone formation indicates the probability that the carbon chain is straight rather than branched. If there were a branch, either the OH or the adjacent non- $\alpha$   $\text{NH}_2$  would be attached to a carbon in the  $\gamma$  position to the carboxyl, and lactone formation would be probable.

*Determination*—The instantaneous formation of a mole of ammonia by action of alkaline periodate is shown not only by hydroxylysine, but also by serine, threonine, and synthetic *dl*- $\beta$ -hydroxyglutamic acid (obtained by courtesy of Dr. H. D. Dakin), and affords a simple analytical means of differentiating these hydroxyamino acids from those which do not have the  $-\text{CH}(\text{NH}_2)-\text{CH}(\text{OH})-$  group. To the amino acid mixture one merely adds periodate and saturated  $\text{K}_2\text{CO}_3$  solution, and determines the ammonia by aeration in the urea apparatus of Van Slyke and Cullen.<sup>6</sup> Cystine, tryptophane, and methionine, as shown by Nicolet and Shinn,<sup>5</sup> also consume periodate oxygen. They do not, however, yield ammonia.

Work on the structure of hydroxylysine is being continued.

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<sup>6</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 211 (1914); **24**, 117 (1916).

**SEPARATION IN NEARLY PURE FORM OF LUTEINIZ-  
ING (INTERSTITIAL CELL-STIMULATING) AND  
FOLLICLE-STIMULATING (GAMETOGENIC)  
HORMONES OF THE PITUITARY GLAND**

*Sirs:*

No satisfactory method of separating pure or nearly pure gonadotropic hormones from the pituitary gland appears to have been published in terms of the following requirements: (1) separation occurs in a single stage without serious loss; (2) the biological potency and purity are established in hypophysectomized immature rats; and (3) there is physicochemical evidence that the hormones are nearly pure. It is believed that the procedures outlined below, if rigidly followed, meet these requirements.

Fresh whole pituitary glands (hog) are fractionated to the last stage of a published method.<sup>1</sup> A salt-free aqueous solution of both hormones (protein N = 4.0 mg. per cc.) is half saturated with  $(\text{NH}_4)_2\text{SO}_4$  and brought to an apparent pH of 4.2. The precipitate contains no activity and is filtered. The protein in the filtrate is precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 0.9 saturation, filtered, and dialyzed until free from sulfate. To 1 volume of the dialyzed solution of hormones are added 1 volume of 1 M acetate buffer, pH 4.41, and 2 volumes of 41 per cent  $\text{Na}_2\text{SO}_4$ .<sup>2</sup> The precipitate is almost pure luteinizing (interstitial cell-stimulating) hormone, whereas follicle-stimulating (gametogenic) hormone remains in solution. Luteinizing hormone is purified to a high degree by repetition of this procedure until the dissolved protein nitrogen is constant (0.025 mg. per cc.). Follicle-stimulating hormone is relatively very soluble in the acetate buffer-sodium sulfate solution and is precipitated by adding about 40 gm. of  $(\text{NH}_4)_2\text{SO}_4$  to each 100 cc. of filtrate. The precipitate is dialyzed until salt-free. All the steps are repeated until a concentrated aqueous solution of

<sup>1</sup> Chow, B. F., Greep, R. O., and van Dyke, H. B., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **126**, P 462 (1939); *J. Endocrinology*, **1**, No. 4 (1940).

<sup>2</sup> The solution must be kept in an incubator and pipetted while warm.

follicle-stimulating hormone is completely free from turbidity when 1 volume of acetate buffer and 2 volumes of sodium sulfate solution are added.

In immature hypophysectomized male rats a total dose of 0.002 mg.<sup>3</sup> causes a detectable gonadotropic effect; luteinizing hormone causes testicular hypertrophy and enlargement of the anterior prostate, whereas follicle-stimulating hormone causes only testicular enlargement. A dose of 0.360 mg.<sup>4</sup> of follicle-stimulating hormone is followed by no enlargement of the anterior prostate. Similarly 0.360 mg.<sup>4</sup> of luteinizing hormone has no effect on the weight of the ovaries or uterus of hypophysectomized immature females. Definite ovarian hypertrophy is produced by 0.010 mg. of follicle-stimulating hormone but not by 0.002 mg.; large doses of this hormone over a period of 10 days cause marked follicular growth with relatively slight change in the weight of the uterus. Histological studies are in progress.

Solubility studies have been made with two solvents<sup>5</sup> for luteinizing hormone and one<sup>6</sup> for follicle-stimulating hormone. There is no evidence of contaminating proteins if the concentration of solid phase is 5 times that saturating the solution, though an increase in the amount of protein nitrogen dissolved is observed if the solid phase is 100 times that necessary for saturation.

We are under great obligation to Dr. John H. Northrop for advice.

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<sup>3</sup> All doses are expressed as mg. of nitrogen; no potentiating agent was used.

<sup>4</sup> Larger doses were not used.

<sup>5</sup> (1) 0.25 M acetate buffer, pH 4.41, containing 20.5 per cent  $\text{Na}_2\text{SO}_4$ ;  
(2) 0.25 M acetate buffer, pH 4.41, containing 15.4 per cent  $\text{Na}_2\text{SO}_4$ .

<sup>6</sup> 0.6 saturation of  $(\text{NH}_4)_2\text{SO}_4$  containing 0.15 M acetate buffer of pH 4.43.

## THE ACTION OF FATTY ACIDS ON THE CHOLINE OXIDASE OF RAT LIVER

*Sirs:*

The relationship of choline oxidase to fat metabolism is still not clear. The enzyme may prevent the accumulation of choline in the liver when phospholipids are being catabolized. On the other hand, if the enzyme is equally active at all times, it is difficult to see how sufficient choline can accumulate for the synthesis of phospholipids from fatty acids. It seemed possible that fatty acids might inhibit the activity of the oxidase and, if so, that the concentration of fatty acid at any time would determine how much free choline would be oxidized and how much would be used for the synthesis of the phospholipids.

The accompanying table shows the effect of stearic acid on the choline oxidase and other oxidases present in the standard rat liver preparation made by a method already described.<sup>1</sup> The

### *Effect of 0.004 M Stearic Acid in Buffer on Activity of Various Oxidases in Rat Liver Preparation*

2.0 mg. of each substrate were used in a total volume of 2.0 cc. in the Warburg vessels. *p*-Phenylenediamine was the substrate for the cytochrome oxidase, proline for the *D*-amino acid oxidase, and isoamylamine for the amine oxidase. The stearic acid was added before the substrates. 0.05 M phosphate buffer, pH 6.7, 37°.

Oxidase	Inhibition
	<i>per cent</i>
Choline.....	61
Succinic.....	19
Cytochrome.....	13
<i>D</i> -Amino acid.....	8
Amine.....	0

percentage of inhibition depends on the relative amounts of acid and enzyme. The choline oxidase is by far the most sensitive.

<sup>1</sup> Bernheim, F., *J. Biol. Chem.*, **133**, 141 (1940).

As the concentration of stearic acid is increased, the succinoxidase is inhibited next and then the cytochrome oxidase. The *d*-amino acid and amine oxidase are inhibited significantly only by much larger concentrations. When stearic acid causes a 60 per cent inhibition, palmitic and oleic acids in equivalent concentrations cause 41 and 29 per cent inhibition respectively. The inhibition occurs only below pH 7.0, indicating that the undissociated acid is the active agent. The initial concentration of choline does not affect the percentage of inhibition. Fats such as olive and cod liver oils and a mixture of cephalin and lecithin are without effect.

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## CARBOHYDRATE CHARACTERIZATION\*

- I. THE OXIDATION OF ALDOSES BY HYPOIODITE IN METHANOL
- II. THE IDENTIFICATION OF SEVEN ALDO-MONOSACCHARIDES  
AS BENZIMIDAZOLE DERIVATIVES

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(Received for publication, November 15, 1939)

In the course of work in this laboratory on several benzimidazoles of the carbohydrate series in 1936, observation was made of their potential value as derivatives for carbohydrate identification. The general chemical and physical properties of the aldo-benzimidazoles appeared to be remarkably superior, from the characterization standpoint, to those of hydrazones and osazones. The following procedure for aldose identification has been developed to utilize the favorable properties inherent in this type of carbohydrate derivative.

The general developmental investigation of methods for the preparation of aldo-benzimidazoles is to be treated separately in order to present concisely in this publication the working procedures for the identification of seven aldo-monosaccharides. Preliminary work has shown, in brief, that the direct oxidative condensation of aldoses with *o*-phenylenediamine, as first studied in 1887 by Griess and Harrow (1), even with the introduction of more recent approaches, fails to give satisfactory yields. The

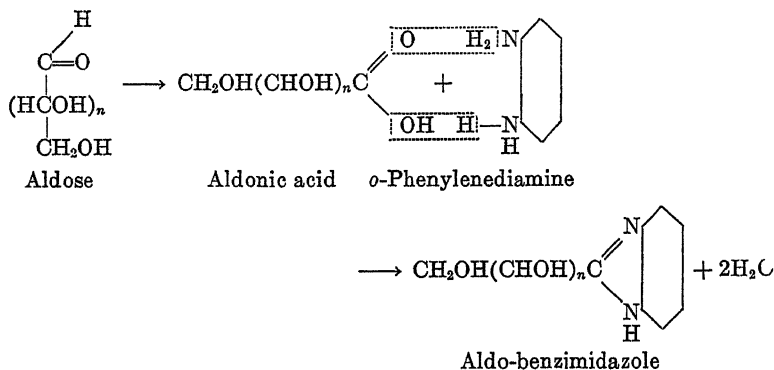
\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Abstracted from a thesis submitted by Stanford Moore to the Graduate Faculty of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1938; Summaries of doctoral dissertations, Madison, 3, 191 (1938).

† Stanford Moore is indebted to the Wisconsin Alumni Research Foundation for a fellowship in 1935-36 and to the Graduate Research Fund for assistantships for 1936-39.



The procedure is based on the combination of (a) a potassium hypiodite-methanol oxidation, and (b) the use of *o*-phenylenediamine as reagent for the characterization of the resulting aldonic acids as benzimidazole derivatives according to the following equations.



For the efficient and rapid preparation of the aldonic acid salts to serve as intermediates in the formation of benzimidazoles, a procedure in which potassium hypoiodite in methanol solution is employed has been developed. In identification work on syrups from natural products we have found these conditions to provide the most convenient laboratory method for obtaining the desired oxidation. However, the products of the bromine-barium benzoate procedure given by Hudson and Isbell (2), the barium hypoiodite oxidation of Goebel (3), or, in general, any oxidation which yields aldonic acids<sup>1</sup> may be identified as benzimidazole derivatives.

The potassium hypoiodite-methanol procedure has been de-

<sup>1</sup> The presence of inorganic salts and organic impurities in aqueous syrups from natural sources generally renders it impossible to take advantage of the efficient electrolytic method for pure aldoses developed by Isbell and Frush (4).

veloped as a simplification of existing methods by the elimination of several operations inherent in other techniques for carrying out the oxidation. The preparation of the potassium salt of gluconic acid in a single operation has been realized by introducing methanol as the reaction solvent for the hypiodite oxidation,  $\text{aldose} + \text{I}_2 + 3\text{KOH} \rightarrow 2\text{KI} + \text{K aldionate} + 2\text{H}_2\text{O}$ . The glucose, iodine, potassium hydroxide, and potassium iodide are all soluble. As the reaction proceeds, the desired potassium gluconate precipitates in the form of anhydrous needles (yield 92 per cent, time 50 minutes). Under the same conditions arabinose and galactose give good yields of the corresponding potassium aldonates. However, when mannose, xylose, rhamnose, or lyxose is oxidized, little or no precipitate is formed because of the appreciable solubility of the potassium salts in the reaction solvent (methanol with about 2 per cent water). Practically complete precipitation of the aldonic acids which thus remain in solution can be accomplished by the addition of a solution of barium iodide in methanol. The barium salts obtained are amorphous, basic, and contain iodide. As intermediates for benzimidazoles, these crude basic salts, rapidly obtained in almost quantitative yields, have proved to be equally as satisfactory as pure barium or calcium salts prepared by longer procedures.

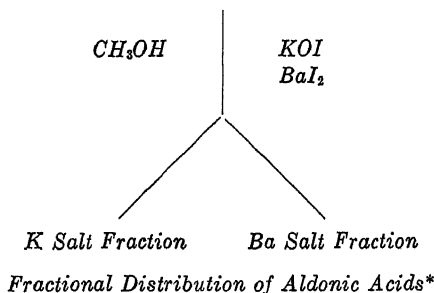
The aldonic acids formed in the oxidation are therefore, in practice, isolated in two separate fractions. The insoluble potassium salts are obtained first. Addition of barium iodide to the filtrate from the potassium salts yields barium salts of the remaining aldonic acids. The partitioning of a 2 gm. sample of a given aldo-hexose (1.7 gm. of pentose) into potassium and barium salts is shown in Table I. It will be noted that the division is relatively sharp between glucose, galactose, and arabinose, and the sugars which precipitate predominantly in the barium salt fraction. In the identification of the aldoses as benzimidazole derivatives, use is made of this preliminary fractionation accomplished in the oxidation step.

In order to determine the optimum experimental conditions for the oxidation, a series of trials on glucose was made involving the variables (a) aldose concentration, (b) ratio of iodine to aldose, (c) rate of potassium hydroxide addition, and (d) temperature. A glucose concentration of 1 to 2 per cent was adopted, together

with the use of twice the theoretical amount of iodine and alkali, dropwise addition of the alkali over a period of about 20 minutes, and a temperature of 40°.

The hypiodite oxidation of aldose to aldonic acid is strictly quantitative only in solutions of lower sugar concentration than was used in this case. The increased carbohydrate concentration results in a small sacrifice in the accuracy of oxidation, as also

TABLE I  
*Aldo-Monosaccharides*



Aldose	Weight of sample	K salt		Weight of Ba salt
		gm.	per cent	
Glucose.....	2.0	2.4	92	0.4
Galactose.....	2.0	2.2	85	0.8
Arabinose.....	1.7	1.9	83	0.5
Mannose.....	2.0	0.9	30	2.5
Xylose.....	1.7	0.2	8	3.4
Lyxose.....	1.7	0.0		3.7
Rhamnose · H <sub>2</sub> O.....	2.0	0.0		3.9

\* The amount of water in the starting syrup, the presence of impurities, and the temperature of the solution at the time of filtration affect the exact extent to which the potassium salts crystallize.

reported by Goebel (3) for the barium hypiodite method. It has not been possible to detect any interference in the benzimidazole method from the trace of overoxidized aldoses present in the potassium and barium salt fractions. It has been readily possible, however, by means of the benzimidazole procedure to detect the small amount of overoxidation by hypiodite in the case of a ketose (fructose), as will be noted in the next section.

Acetone-free methanol is preferable as solvent but the content

of this impurity in commercial absolute methanol is frequently low enough (0.1 per cent) to cause no serious interference. A blank oxidation (no aldose present) on the given experimental scale should cause little or no precipitation. Under normal conditions a slight precipitate may form (50 to 70 mg.), a part of which is potassium or barium carbonate. Methanol may also contain reducing impurities which consume hypoiodite but give no precipitable reaction products. The most practical control on the reagents is an oxidation on crystalline glucose which should give a 90 per cent yield of potassium gluconate.

The operations involved in this oxidation can be conveniently performed on a smaller scale than given here for 2 gm. samples. On small quantities the reaction itself can be run in a centrifuge tube to minimize transfer losses.

#### EXPERIMENTAL

*Sample for Oxidation*—In testing the procedure on known crystalline sugars, prepare a concentrated sugar solution by dissolving 2 gm. of aldo-hexose (1.7 gm. of pentose)<sup>2</sup> in 3 cc. of water. In the oxidation of samples from natural products, concentrate the carbohydrate fraction to a syrup and make a Willstätter-Schudel titration (5) on a few tenths of a gm. of this syrup. Weigh out a sample equivalent to 2 gm. of aldo-hexose. If the syrup is thick, add 1 or 2 cc. of water to the sample with warming until it flows readily. As an alternate procedure with dilute carbohydrate solutions, a volume of solution equivalent to 2 gm. of aldo-hexose can be taken and concentrated directly under reduced pressure to about 4 cc.

#### *Reagents*—

Absolute methyl alcohol (acetone-free).

Solution of potassium hydroxide in methanol (40 gm. per liter).

Resublimed iodine.

*Apparatus*—A 3-necked flask (500 cc.), equipped with a stirrer, thermometer, and dropping funnel, is half immersed in a water bath warmed by steam (hood). For filtration, a Buchner funnel is used in the center neck.

*Oxidation*—Add 5.7 gm. of iodine and 80 cc. of methanol to the flask and stir for a few minutes. Warm the water bath to

<sup>2</sup> The method of oxidation is also applicable to uronic acids and disaccharides.

bring the methanol solution to about 40° and apply no more heat during the oxidation.

Warm the sample to be oxidized (aldo-hexose content of 2 gm., volume of about 4 cc.) on a steam bath and dissolve in 25 cc. of methanol. Filter the solution (if not perfectly clear) by suction through asbestos directly into the iodine solution in the flask. Use decolorizing carbon if the sugar solution is dark. Rinse onto the filter with two 10 cc. portions of methanol. With pure aldoses the addition of methanol causes no precipitation and the solution is poured directly into the flask without filtration.

As soon as the sugar has been added to the iodine and methanol, replace the stirrer and start the dropwise addition (over a period of 10 to 15 minutes) of 65 cc. of 4 per cent potassium hydroxide

TABLE II  
*Composition of Crude Potassium Salts*

Unpurified potassium salts	M.p.	$[\alpha]_D^{25}$	Potassium	
			Calculated	Found
	°C.	degrees	per cent	per cent
<i>l</i> -Arabonic.....	211 (with decomposition)	+4.5	19.1	20.3
<i>d</i> -Galactonic.....	164    "    "	+4.3	16.7	16.9
<i>d</i> -Gluconic.....	180    "    "	+11.0	16.7	16.8

solution. Stir for 10 minutes and add dropwise 50 cc. more of potassium hydroxide solution. The final color of the reaction mixture should be a light straw-yellow, indicating removal of nearly all of the free iodine. A few more cc. of alkali may be added if the iodine color is still prominent. Stir for a final 10 minutes.

*Potassium Salts*—Remove the flask from the water bath and cool to room temperature before filtering the potassium salts onto a thick paper on a Buchner funnel under gentle suction. Wash twice with methanol and once with ether. Air-dry. In the characterization procedure, in order to eliminate the possible loss of a component acid in the process of recrystallization, the potassium salt fraction is used directly, without purification for benzimidazole preparation. The constants shown in Table II

indicate the composition of the crude salts from arabinose, galactose, and glucose. Potassium carbonate is the principal inorganic impurity.<sup>3</sup>

*Barium Salts*—Return the filtrate from the potassium salt precipitation to the reaction flask and add a solution of 5 gm. of barium iodide (dihydrate) in 25 cc. of methanol dropwise with stirring.<sup>4</sup> Centrifuge the precipitated basic barium salts, wash twice with  $\text{CH}_3\text{OH}$  on the centrifuge, suspend in ether, and transfer to a Buchner funnel under slight suction. Air-dry for about 10 minutes and remove the final traces of solvents in a vacuum desiccator. If left in the air for too long a period the salts occasionally become hygroscopic. Analysis of the crude

TABLE III  
*Composition of Crude Barium Salts*

Crude barium salts	Barium	Barium per mole aldose
	<i>per cent</i>	<i>moles</i>
<i>d</i> -Lyxonic.....	37.9	0.90
<i>d</i> -Mannonic.....	35.7	0.95
<i>l</i> -Rhammonic.....	39.3	1.02
<i>d</i> -Xylonic.....	39.6	0.95

salts (Table III) shows them to contain approximately 1 molecule of barium per molecule of aldose represented by the salt (calculated in conjunction with the data on fractional distribution).

<sup>3</sup> For preparatory purposes the pure potassium aldonates may be obtained by recrystallization of the unpurified salts from water (a minimum) and methanol or ethanol.

<sup>4</sup> If no appreciable potassium salt precipitation occurs, filtration is omitted and the barium iodide is added directly to the oxidized solution. Also the barium iodide can be added before the oxidation if no potassium salt fraction is desired, as is the case when the oxidation is being used for preparative purposes on a known aldose such as mannose. With the barium added at the start, 2 gm. of mannose yield 4.1 gm. of barium salt. The barium precipitates obtained in this manner are fairly granular and filter faster than the somewhat gelatinous salts formed by the addition of barium iodide at the end of the reaction. The salts obtained by both methods show essentially the same composition.

## *II. The Identification of Seven Aldo-Monosaccharides As Benzimidazole Derivatives*

The 2-(aldo-polyhydroxyalkyl)benzimidazole derivatives<sup>5</sup> are stable white crystalline solids, with sharp melting points and general chemical properties which make them specially suitable for identification work.<sup>6</sup> The fundamental operations involved in their preparation and isolation are simple and rapid. They can be prepared in 50 to 80 per cent yields by heating the aldonic acid with *o*-phenylenediamine, preferably in the presence of acid catalysts.<sup>7</sup> The condensation is best carried out in most cases at an oil bath temperature of 135° with the use of hydrochloric and phosphoric acids. (Xylonic acid requires a higher temperature of 180° in the presence of zinc chloride.) In the isolation of the derivatives, advantage is taken of their slight solubility in water and dilute ammonium hydroxide, high solubility in aqueous hydrochloric acid, and insolubility in acetone and ether. Aqueous acid is also conveniently employed as the solvent in determining the specific rotations.

In contrast to the behavior of many of the derivatives of carbohydrates, the benzimidazoles crystallize readily. Individual derivatives may be separated by fractional crystallization. Practical examples are the rapid identification of glucose and arabinose in the same sample, or mannose, galactose, and glucose occurring simultaneously.

Two properties to be desired in an organic characterization

<sup>5</sup> The imidazole structure for the carbohydrate derivatives was established by Hinsberg and Funcke (6) in 1893 and confirmed by Ohle (7) in 1934.

<sup>6</sup> It is interesting to note that Schilling (8) in 1901 wrote of the possible superiority of carbohydrate benzimidazoles over osazones as characterization derivatives but he was unable to utilize the idea because of the low yields.

<sup>7</sup> In the preparation of a series of aldo-benzimidazoles for studies in the resolution of racemic acids, Haskins and Hudson (9) in 1939, completely independently of our work, have employed the aldonic acid condensation (100° both with and without addition of HCl) to give good yields in a number of cases. Their application of the benzimidazole from *D*-gluco-*D*-gulo-heptonolactone to the practical preparation of *L*-tartaric acid makes use of the crystallization properties of the salts of this type of heterocyclic base.

derivative are (1) a means for quantitative isolation and (2) the possibility of secondary derivative formation. The aldo-benzimidazole possesses both of these properties. The fact that benzimidazoles in general can be precipitated as copper salts from aqueous solution facilitates the isolation of the small amounts of derivatives which may remain in solution after direct crystallization. The benzimidazole is regenerated from the copper salt by the use of hydrogen sulfide. The potential value of this property of the benzimidazole nucleus in identification work is evident. It means, for example, that with a sample containing a small amount

TABLE IV  
*Benzimidazole Derivatives of Aldo-Monosaccharides*

Carbohydrate	Benzimidazole*		Hydrochloride m.p.	Picrate m.p.
	M.p.	$[\alpha]_D^{25}$		
	°C.	degrees	°C.	°C.
<i>l</i> -Arabinose.....	235†	+49.2	230	158
<i>d</i> -Galactose.....	245†	+43.3	202-204	217†
<i>d</i> -Glucose.....	215	+9.6	180	203†
<i>d</i> -Lyxose.....	189	-12.8	191	95-99
<i>d</i> -Mannose.....	227†	-22.0	101-150	205†
<i>l</i> -Rhamnose.....	207	+27.4	173-175	168
<i>d</i> -Xylose.....	224	+64.8	200-202	191

\* All melting points are uncorrected. The rotations were taken in 5 per cent citric acid solution with  $c = 2$  (approximately).

† With decomposition.

of glucose in the presence of a relatively large quantity of arabinose, the gluco-benzimidazole in the mother liquors will be recovered by copper salt precipitation and will not escape detection.

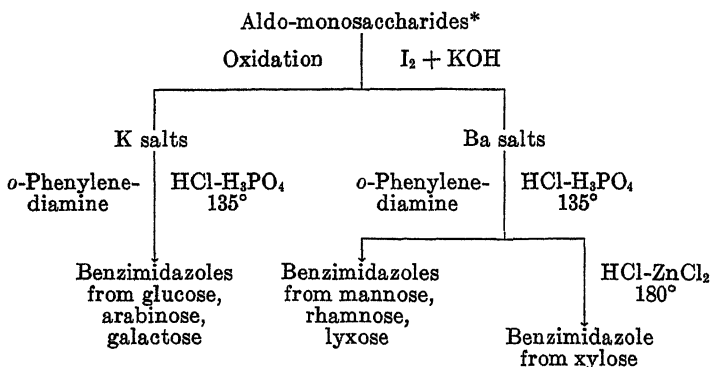
Secondary derivatives of the benzimidazole nucleus can be prepared by making crystalline acid salts which provide third and fourth constants for identification. The hydrochlorides and picrates are readily obtained and in general have characteristic melting points which are lower than those of the parent base.

When three or four of the constants have been taken on any aldo-benzimidazole in Table IV, the identity of the derivative is known beyond any question of doubt. This accuracy fills a need in carbohydrate chemistry which an osazone, or any other single



derivative, does not satisfy. The two constants (m.p. and  $[\alpha]_D$ ) which can be taken on a hydrazone or osazone are limited in accuracy, as those who have worked with them well know, by anomalous melting behavior and by mutarotation of the difficultly soluble derivatives in the colored solutions which they give in pyridine and alcohol. The aldo-benzimidazoles are free from these disadvantages. In the case of the benzimidazole derivatives it is not necessary to resort to description of crystalline habits as a means of identification.

The chemical properties of the benzimidazole nucleus which contribute to its value as a tool for characterization are best made apparent by the experimental detail of the identification procedure.



\* This schematic presentation of the procedure is to be considered in connection with Table I of the potassium hypoiodite-methanol oxidation method, which gives the partitioning of aldonic acids between the two salt fractions. Not indicated in the diagram, for instance, is the fact that while 90 per cent of the benzimidazole from a pure glucose sample will appear in the potassium salt fraction, the residual 10 per cent is also detectable in the barium salt fraction.

#### EXPERIMENTAL

*Condensation with Gluconic Acid*—A specific example of aldo-benzimidazole preparation is of illustrative value. Calcium gluconate is a convenient starting product. To 2 gm. of Ca gluconate·H<sub>2</sub>O (0.009 mole of gluconic acid) in a test-tube, add 1.1 gm. (0.01 mole) of *o*-phenylenediamine, about 4 cc. of water, 1 cc. of ethanol, and 1.7 cc. (0.02 mole) of concentrated

hydrochloric acid.<sup>8</sup> Warm until in solution, add a boiling chip, and heat for 2 hours on an oil bath kept at  $135^{\circ} \pm 5^{\circ}$ . The tube should be immersed to about the level of the contained solution. During the 1st hour water boils off to leave a thick syrup in which bubbling gradually ceases during the 2nd hour. Remove the tube from the bath and while still warm dissolve the syrup in about 10 cc. of water, add carbon, filter through asbestos, dilute the filtrate to about 30 cc., and make alkaline with ammonium hydroxide. Crystallization is usually rapid. When it is complete, filter and wash the crystals with water, acetone, and ether. The yield of gluco-benzimidazole (m.p.  $215^{\circ}$ ) is 1.7 to 1.9 gm. (70 to 80 per cent of theory).

*Condensation from Potassium Salts*—For the identification of the aldonic acids present in the potassium salt fraction of the potassium hypiodite-methanol oxidation, 1 gm. of potassium aldinate is added to 0.6 gm. of *o*-phenylenediamine, 0.8 cc. of concentrated HCl, 0.4 cc. of syrupy  $\text{H}_3\text{PO}_4$  (sp. gr. 1.7), 2 cc. of water, and 1 cc. of ethanol. Carry out the condensation as given above for Ca gluconate. Use 5 cc. of water to dissolve the syrup and crystallize from a volume of approximately 15 cc.

If the tube is too large in reference to the size of sample, the small syrupy residue may spatter on the wall of the tube and fail to undergo complete condensation. To avoid this, in experiments with small quantities, the addition of a few tenths of a cc. of diethylene glycol at the start of the reaction has been found helpful in insuring smooth condensation.

When the starting products are relatively pure aldoses, crystallization of the resulting benzimidazoles is usually rapid. The yield is 0.7 to 0.9 gm. (60 to 80 per cent of theory). If crystallization is slow, leave the flask in a refrigerator for 12 hours.<sup>9</sup> In the absence of crystals, filter off any trace of amorphous precipitate

<sup>8</sup> With calcium salts good yields are obtained without the use of phosphoric acid in addition to hydrochloric. If phosphoric acid is used the calcium must be removed first to prevent the formation of a calcium phosphate precipitate when the reaction mixture is made basic.

<sup>9</sup> If too large an excess of ammonium hydroxide has been added, ammonium phosphate may crystallize from the reaction mixture. If removed by filtration, the crystals dissolve readily when washed with water and offer no interference.

which may have formed and extract the filtrate with ether three times to remove unreacted *o*-phenylenediamine. Mother liquors from a first crystallization are also submitted to ether extraction.<sup>10</sup> Evaporate the extracted solutions nearly to dryness. Take up in a few cc. of water and filter off any water-insoluble crystals. They may represent a first crop or a second crop from the mother liquors.

*Copper Salt Precipitation*—To the filtrates, from which excess ammonia has thus been removed by the evaporation, add a cupric ammonia solution prepared by suspending 10 gm. of cupric acetate (monohydrate) in water, adding sufficient  $\text{NH}_4\text{OH}$  to give a clear solution, and diluting to 100 cc. Estimate approximately the amount of benzimidazole in the mother liquor and add about 20 cc. of cupric ammonia solution per gm. of benzimidazole. If a benzimidazole is present, a heavy green precipitate forms. Warm the mixture on a steam bath for 5 to 10 minutes with an air jet directed on the surface of the solution to insure complete precipitation (removal of excess  $\text{NH}_4\text{OH}$ ). Transfer to a tube and wash with water three times on the centrifuge. Suspend the washed salt in 4 to 5 times its volume of 3:1 water-alcohol, decompose with hydrogen sulfide, heat and aerate, centrifuge while warm, and decant from the copper sulfide through a carbon-asbestos pad under suction.

The benzimidazoles in the filtrate (usually a few tenths of a gm.) thus are separated from practically all contaminants and crystallize readily upon concentration of the solution by evaporation. After the evaporation add 1 or 2 drops of  $\text{NH}_4\text{OH}$  to insure alkalinity.

*Condensation from Barium Salts*—Weigh out a sample of barium salt equivalent to about 1 gm. of aldo-hexose (approximately 2 gm. of salt in the absence of contaminants), suspend in 10 cc. of water in a centrifuge tube, add phenolphthalein, and neutralize by the addition of dilute  $\text{HCl}$ . To the neutral solution

<sup>10</sup> With unknowns it is frequently of value to transfer the ether extract to a tared evaporating dish for determination of the weight of ether-extractable solids. The recovery of *o*-phenylenediamine thus estimated gives an indication of the amount which has undergone reaction. A condensation of potassium gluconate (1 gm.) as above yields an ether extract of 0.1 gm. (theoretical excess of *o*-phenylenediamine added, 0.14 gm.).

add 0.8 cc. of concentrated HCl and precipitate the barium ion (approximately 0.5 cc. of 1:1 concentrated sulfuric acid-water). On the basis of drop tests, adjust the solution until it shows a slight excess of barium. An excess of sulfuric acid is to be avoided. Centrifuge, decant through a filter, and evaporate the filtrate (steam and air) to about 4 cc. Transfer to a test-tube (using ethanol in washing) containing 0.7 gm. of *o*-phenylenediamine. Add 0.5 cc. of  $\text{H}_3\text{PO}_4$  and condense as in the case of the potassium salt. If foaming occurs during the early part of the condensation, a few drops of ethanol may be added to wash down the material on the sides of the tube by reflux.

In working up the condensate, at the time of addition of  $\text{NH}_4\text{OH}$ , filter the solution rapidly through a carbon-asbestos pad within a few seconds after the addition of ammonia in order to remove the amorphous precipitate which may form. If the filtration is not performed rapidly, the relatively insoluble benzimidazole of mannose may begin crystallization during the filtration. On direct crystallization, the yields are 0.7 to 0.8 gm. (50 per cent of theory) of benzimidazole per gm. of aldose involved. Extract the mother liquors with ether and work up by the copper salt procedure to obtain the derivatives remaining in solution.

*Condensation at 180°*—Under the above conditions at 135° xylonic acid yields little or no benzimidazole derivative. Completion of the condensation can be accomplished at 180°. The difference in reactivity of the individual aldonic acids toward *o*-phenylenediamine can therefore in this case be used for the purpose of separation. The solution obtained by decomposition of the copper salt with  $\text{H}_2\text{S}$  contains the water-soluble intermediate reaction product with *o*-phenylenediamine of any xylonic acid present. The solution is evaporated to a thin syrup as usual to permit crystallization of any benzimidazoles of other aldoses present.

Transfer the syrup to a test-tube and, for an estimated 0.5 gm. as the possible xylose content,<sup>11</sup> add 0.5 cc. of concentrated HCl and 0.3 gm. of zinc chloride (preferable to  $\text{H}_3\text{PO}_4$  in this case).

<sup>11</sup> This estimate (1, 0.5, 0.25, etc., gm.) of the xylose content, if any, for the purpose of determining the approximate amounts of reagents to be added, can be roughly made on the basis of the amounts of benzimidazoles of other aldoses already isolated and pentose tests on the original sample.

Place on the oil bath at  $135^{\circ}$  and raise the temperature over a period of 45 minutes to  $180^{\circ} \pm 5^{\circ}$  and hold at that temperature for 1 hour. Take the condensed syrup up in water as usual and decolorize. The addition of  $\text{NH}_4\text{OH}$  causes precipitation of zinc benzimidazole salts and zinc hydroxide, both of which are soluble in excess  $\text{NH}_4\text{OH}$ . But in this case it is preferable to add a minimum of  $\text{NH}_4\text{OH}$ , remove the excess by heat and air as in the copper procedure, pass  $\text{H}_2\text{S}$  directly into the resulting zinc salt suspension, heat, and centrifuge while warm. Decant from the zinc sulfide through a filter, and free from hydrogen sulfide. Xylo-benzimidazole, if present, crystallizes during concentration of the filtrate. Add 1 or 2 drops of  $\text{NH}_4\text{OH}$  at the end of the evaporation. In this procedure, 1 gm. of xylose in the original sample will yield about 0.4 gm. of benzimidazole. The yield is higher (0.7 gm., 50 per cent) if the  $180^{\circ}$  condensation is run directly on the barium salt without the intermediate steps involved above. In the absence of appreciable quantities of other aldonic acids in the barium fraction, the direct high temperature condensation can be run on a separate sample of barium salt. A weight equivalent to 1 gm. of aldose is taken, which is 2 gm. in case of the usual salt, neutralized, 1 cc. of concentrated  $\text{HCl}$  added, and the solution freed of barium and condensed with 0.7 gm. of *o*-phenylenediamine and 0.5 gm. of zinc chloride, as given above, at  $180^{\circ}$  for 1 hour.<sup>12</sup>

*Behavior of Fructose*—This ketose has been carried through the benzimidazole scheme to determine what reactions, if any, it undergoes. We do not present the following data as an efficient method for fructose identification but as information of value in the application of the benzimidazole procedure for the identification of aldoses to a natural product also containing fructose. 2 gm. of fructose, when put through the potassium hypoiodite-methanol oxidation, yield practically no precipitate in the potassium salt fraction. A barium salt precipitate of 2.9 gm. is obtained which gives a strong Seliwanoff's test for fructose. Condensation of this barium salt fraction by the same procedure given

<sup>12</sup> The benzimidazoles which form readily at  $135^{\circ}$  are not all stable at the higher temperature of  $180^{\circ}$ . Therefore xylo-benzimidazole is at present the only product of the  $180^{\circ}$  condensation which possesses characterization value.

for aldoses produces considerable charring during the period of heating. The acidic filtrate from the first decolorization is almost black. The addition of  $\text{NH}_4\text{OH}$  precipitates the dark impurities as a tar which is removed by the filtration of the alkaline solution to give a final clear filtrate. On standing, or during the process of ether extraction, the filtrate deposits a small amount (0.1 gm.) of crystals and concentration of the solution yields a second crop (0.1 gm.) of the same product (m.p.  $235\text{--}236^\circ$ ,  $[\alpha]_{\text{D}}^{25} = -51^\circ$ , hydrochloride m.p.  $229^\circ$ , picrate m.p.  $158^\circ$ ). The compound thus proves to be *d*-arabo-benzimidazole.

It can be stated, therefore, that the presence of fructose does not interfere with the isolation of aldo-benzimidazoles. It has no effect on the potassium salt fraction and the unchanged fructose in the barium salt undergoes decomposition in the presence of the mineral acids to give hydroxymethylfurfural and other products which, either as such or in condensed forms with *o*-phenylenediamine, are readily removed from the benzimidazoles by the alkaline filtration. The only precaution to be taken is in the interpretation of the isolation of *d*-arabo-benzimidazole. Since *l*-arabinose is the form which usually occurs in nature, the cases for possible confusion are rare. But if the *d* form of arabinose is present in appreciable quantity in a natural product, the corresponding derivative will be readily identified from the potassium salt fraction. The problem of whether a small amount of crystals of *d*-arabo-benzimidazole present in the products from the barium salt condensation may have come from *d*-arabinose, *d*-fructose, or both, should prove solvable in conjunction with pentose and ketose tests on the sample and the methylphenylhydrazine reaction.

The isolation of the pentose derivative from a ketose indicates that oxidation of the terminal  $\text{CH}_2\text{OH}\text{--CO}$  grouping has taken place to some extent during the hypoiodite oxidation. Pure fructose (unoxidized), when submitted to the condensation conditions with *o*-phenylenediamine and  $\text{HCl}\text{--H}_3\text{PO}_4$ , gives no *d*-arabo-benzimidazole.

*Recrystallization*—The aldo-benzimidazoles can be recrystallized and fractionated from hot water. The relative solubilities may be indicated by giving the approximate volume of hot water required to recrystallize 1 gm. of the given derivative. For

lyxo-benzimidazole 15 cc. are required, for gluco- 20, rhamno- 30, xylo- 40, manno- 100, and arabino- and galacto- about 200. In recrystallizing the last three derivatives it is preferable to use 1:1 ethanol-water in which they are more than twice as soluble as in water alone. With these less soluble derivatives it is also convenient in some cases to suspend the derivative in a small volume of hot water, to add dilute HCl until the benzimidazole is in solution, filter, and add  $\text{NH}_4\text{OH}$  to the reheated filtrate. It is always satisfactory to use HCl in the first recrystallization but for a sample being crystallized for specific rotation or analysis no acid should be used, since this treatment may not remove small amounts of acid-soluble, alkali-insoluble impurities.

*Analyses*—The rhamno, lyxo, and xylo derivatives (Table V) have not been reported in the previous literature (1, 7, 9).

TABLE V  
*Analyses of Rhamno, Lyxo, and Xylo Derivatives*

Benzimidazole	Nitrogen (Dumas)	
	Calculated	Found
	<i>per cent</i>	<i>per cent</i>
<i>l</i> -Rhamno-.....	11.11	11.0
<i>d</i> -Lyxo-.....	11.77	11.8
<i>d</i> -Xylo-.....	11.77	11.8

*Preparation of Picrates*—Suspend about 100 mg. of pure aldo-benzimidazole in 1 cc. of water and add an equal weight of picric acid (containing 15 per cent water). If the picric acid salt does not completely dissolve when the mixture is warmed, add ethanol dropwise. Cool, filter the crystals on a small Buchner funnel, and wash with water. When the starting benzimidazole is pure, the melting point of the picrate is usually correct without further purification. If required, recrystallize from water and ethanol. In all cases, especially with semimicro quantities, a reasonably accurate weighing of the benzimidazole and its picric acid equivalent is desirable.

*Preparation of Hydrochlorides*—To 15 mg. of pure aldo-benzimidazole in a 3 cc. centrifuge tube, add 0.1 cc. of dry HCl in absolute ethanol (about 10 per cent HCl by weight). On slight warming the benzimidazole dissolves and may almost simul-

taneously crystallize out as the hydrochloric acid salt. If crystallization occurs at this point, stir for a few minutes to insure complete reaction. To the solution (or crystal suspension) add 0.5 cc. of acetone. If necessary, cool with scratching to induce crystallization. In some cases the further addition of 1 cc. of ether may be required. Wash the hydrochloride (centrifuge) with 0.5 cc. of acetone followed by 0.5 cc. of ether, and air-dry. The hydrochlorides can be recrystallized from ethanol and acetone. The melting points of the hydrochlorides of most benzimidazoles are sharp and are usually lower than the melting point of the parent base. The manno salt, as obtained, is hydrated and shows a characteristically low melting range. Gluco-benzimidazole hydrochloride,  $C_8H_{17}O_5N_2Cl$ , calculated, Cl 11.66; found, Cl 11.7.

*Specific Rotations*—Benzimidazoles dissolve in aqueous acids to give colorless, non-mutarotating solutions (in contrast to osazones). Hydrochloric acid (1 to 5 N) has been used by others (7, 9) as solvent. In order to avoid repeated use of HCl with polariscope tubes and accessories, we have considered it preferable to choose a non-corrosive acid. A 5 per cent aqueous solution of citric acid has been adopted for the benzimidazole rotations reported here. The solution of citric acid is filtered through sintered glass before being used as solvent for the rotations and it is checked ( $[\alpha]_D = 0.00^\circ$ ) to be sure that it is free from optically active impurities.

It is to be noted that the rotation observed in citric acid may not be identical with the value obtained in a different acid solvent. Galacto-benzimidazole, for example, gives a specific rotation of  $43.3^\circ$  in citric acid and  $44.4^\circ$  in hydrochloric. With gluco-benzimidazole the difference is negligible (in citric acid,  $9.6^\circ$ ; in hydrochloric,  $9.4^\circ$ ).

#### DISCUSSION

The fact that the benzimidazole procedure is not now applicable to aldo-disaccharides and ketoses and has not yet been studied with all aldo-monosaccharides places a limit on its present range of use. The practical value of the benzimidazole derivatives can be utilized directly for the seven aldoses presented here and in other cases may prove of value in supplementary relationship with other characterization reactions in the sugar group. Ex-



tension of the benzimidazole procedure is being made to include additional aldoses, members of the uronic acid series, and the characterization of naturally occurring aldonic acids in the presence of aldoses. The operations in the procedure can be conveniently carried out on a smaller scale than that given here and a description of the detail of semimicro application is in preparation.

In the hydrazone and osazone reactions the purity of the sugar sample and of the hydrazine reagent is a major factor in determining satisfactory results. It is of practical significance that in general the benzimidazole reaction is much less sensitive to the presence of impurities. We have not yet encountered crude aldose syrups from which the benzimidazoles so far studied could not be prepared in satisfactory yields. It may be mentioned, also, that the reagent for the benzimidazole reaction is more convenient to handle than phenylhydrazine. *o*-Phenylenediamine is a solid, and commercial preparations can be used without further purification.

The separation of carbohydrate components from oxidizable and non-oxidizable materials which may be present in a natural sample is accomplished in certain cases by the hypiodite-methanol oxidation. In general, the addition of methanol and filtration into the reaction flask may remove methanol-insoluble impurities from the aldoses. As the oxidation proceeds, the precipitation of the salts of the aldonic acids, in turn, separates the carbohydrate portion from methanol-soluble material. Impurities may be present in some instances which will tend to come out as gums on the salt fractions. It has been observed that lead acetate clarification of the original aldose sample may remove contaminants of this type. Organic acids, if present, may also precipitate as potassium or barium salts. In the identification procedure, the benzimidazole derivatives resulting from most aliphatic and aromatic acids are soluble in alcohol and ether and therefore can be separated from the carbohydrate derivatives which are all relatively insoluble in these two solvents.

#### SUMMARY

A method has been developed for the hypiodite oxidation of aldoses to aldonic acids in methanol. High yields of the potas-

sium or barium aldonates are obtained in one operation by precipitation from the reaction mixture (yield 90 per cent or over, oxidation period 50 to 60 minutes). This oxidation procedure has been developed not as a preparational method for chemically pure aldonic acid salts but efficiently to provide satisfactory salts to serve as intermediates in a method of aldose identification based on the characterization of the component aldonic acids as benzimidazole derivatives.

The condensation of aldonic acids with *o*-phenylenediamine to give 2-(aldo-polyhydroxyalkyl)benzimidazoles has been developed as the basis for a systematic procedure for the identification of seven aldo-monosaccharides in natural products. The chemical and physical properties of the aldo-benzimidazole derivatives have been found to be remarkably superior, from the characterization standpoint, to those of hydrazones and osazones. A description has been given of the preparation, properties, and use of these derivatives in the characterization of arabinose, galactose, glucose, lyxose (if naturally occurring), mannose, rhamnose, and xylose.

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## A SMALL APPARATUS FOR EXTRACTING URINARY ANDROGENS\*

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A series of clinical studies in progress in this laboratory has necessitated a large number of hormone extractions. Using the various apparatus available at the present time, we experienced no particular difficulty with any of them when only a limited number of experiments were desired. However, decided difficulties arose when large scale determinations were attempted.

Gallagher, Koch, and Dorfman (1) used a large apparatus in extracting androgens from human urine by the use of benzene. In making biological assays to determine the androgenic activity they used the intramuscular injection method on capons, which required a large amount of the extract. Fussgänger (2) described a comb inunction assay for androgenic activity in capons. Zimmerman (3) described a colorimetric reaction for the determination of hormone content in the urine. Oesting and Webster (4), using the Fussgänger method and the Zimmerman reaction, roughly correlated these two methods. Callow, Callow, and Emmens (5) made a thorough investigation of the colorimetric method for the measurement of the 17-ketosteroids. They found that the biological activity of androgens is associated with the ketonic fraction of extracts of hydrolyzed urine. From these facts they justified the use of the colorimetric reaction for estimating the excretory transformation products of male hormones.

In the clinical study undertaken we are using the colorimetric method as modified and improved by the above authors (5). These colorimetric findings were verified at frequent intervals by

\* Aided by a grant from the Schering Corporation, Bloomfield, New Jersey.

using the inunction method of assay on capons. Since in either the colorimetric method or the inunction assay only a comparatively small amount of the extract is necessary, there seemed to be a place for a small extractor which not only would be as efficient as the large apparatus but would adapt itself to easier handling and require smaller amounts of urine. The extractor therefore allows experimental work on smaller animals than have been used up to the present time.

The apparatus developed in this laboratory is essentially a modified Soxhlet extractor, the change consisting mainly in bringing the siphon tube to a level high enough to permit the extraction fluid to drain back into the flask below.

A Pyrex extraction apparatus No. 3880M<sup>1</sup> is used (Fig. 1) with a 300 ml. flat bottom flask (*A*) fitted to an extractor jacket (50 × 250 mm.) (*B*) by a ground glass joint. As previously mentioned, the siphon tube (*C*) reaches a level about 5½ inches above the base of the extraction jacket. In the place usually occupied by the extraction thimble a funnel arrangement is inserted which allows the condensate to run from the delivery tube of the condenser to the bottom of the extractor jacket. From there the fluid rises in a continuous stream of finely divided globules through the material to be extracted. We use two means to accomplish this necessarily fine dispersion of extraction liquid. One means consists of fusing a sintered glass disk to the lower end of the funnel tube (*E*). This disk is obtainable as a gas filter tube (Jena glass filters, pattern No. 33c, porosity G0). A simpler and more economical way consists in using a thistle tube (*F*) for the funnel and attaching to it an Aloxite brand aeration stone, cone-shaped and of coarse grit (No. N8D2, Carborundum Company, Niagara Falls). It was found that a stiff paste made of glycerol and litharge will fasten the stone permanently. Should the tube be broken, the residual glass can be removed from the hole in the stone by mechanical means and a new thistle tube sealed in. The stone disperser is less costly and obviates the necessity of expert glass work.

The extractor jacket is fitted by a ground glass joint to a Friedrich type condenser (*D*) in which the delivery tube is cut or

<sup>1</sup> Fisher Scientific Company, Pittsburgh.

chipped to about one-quarter of its original length. This is done in order to give the inner funnels greater height, thereby increasing the hydrostatic pressure of the fluid within the funnel, which in turn accelerates the flow of the extracting fluid.

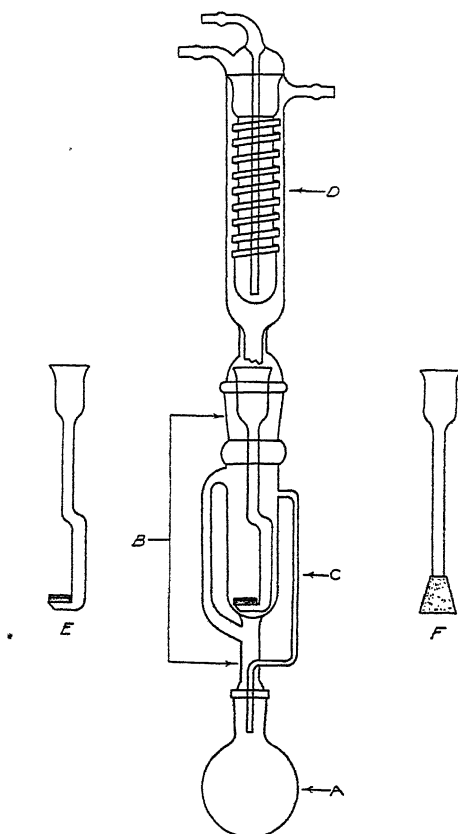


FIG. 1. Extraction apparatus

The apparatus can be obtained in four sizes, 50, 85, 200 ml., and an extra large size of 500 ml. (No. 3880M, Fisher Scientific Company, Pittsburgh). It is likely that the 50 ml. and 85 ml. volumes will be found too small. The 200 ml. size is being used in our present work and is entirely satisfactory. It is large enough

for about 250 ml. of urine, the exact amount depending on the method used for hydrolysis.

We have verified the findings of Gallagher, Koch, and Dorfman (1) and find that the treatment of a hydrolyzed urine sample in a continuous extractor for a period of 3 hours with benzene results in a complete removal of all androgenic material. The efficiency of our extractor was studied by comparison with the Koch continuous extractor (6). The respective recoveries are presented in Table I.

It will be seen that the small extractor shows the same efficiency in less time ( $2\frac{1}{2}$  hours) than is required by the Koch method. It was found that an extraction period of less than  $2\frac{1}{2}$  hours did not

TABLE I  
*Comparison of Maximum Yields of "Sterone"*

Koch continuous extractor (3 hrs.)	Extractor described ( $2\frac{1}{2}$ hrs.)
<i>mg. per l.</i>	<i>mg. per l.</i>
1.08	1.35
3.02	3.00
3.03	3.33
3.11	3.00
2.16	2.26
2.15	2.20
11.84*	11.76*

\* 5 day urine sample.

give the maximum yield. It has been estimated that from 1200 to 1500 ml. of benzene pass through the volume of urine used in about  $2\frac{1}{2}$  hours.

While we are using benzene in the present work, studies in progress in this laboratory indicate the advisability of using ether.

The practicability of this simple extractor has been verified by clinical results which will be published later, in an extensive series of clinical studies made in collaboration with Dr. Walter M. Kearns, Department of Urology, Marquette University School of Medicine.

#### SUMMARY

A small extraction apparatus for hormone work is described. The advantages claimed lie in the simplicity of construction and

the increased efficiency in performance and handling. The cost is much lower than that of other apparatus designed for such work and all parts are interchangeable.

Our thanks are due to the Schering Corporation, to Dr. Max Gilbert of the Schering Corporation for the supply of experimental androgens, to Dr. Walter M. Kearns of the Department of Urology for his aid and close cooperation, and finally to Dr. Joseph C. Bock, Director of the Department of Biochemistry, Marquette University School of Medicine, for advice and encouragement.

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## RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

### X. THE PHOSPHOLIPID TURNOVER OF FRATERNAL TUMORS\*

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Radioactive phosphorus was employed in a recent study to determine the rate of phospholipid turnover in four types of transplantable tumors: a mammary carcinoma, a lymphoma, a lymphosarcoma, and Sarcoma 180.<sup>1</sup> From the rate at which labeled phospholipid is deposited after the administration of  $P^{32}$ , it was concluded that the phospholipid metabolism of tumors bears a greater resemblance to that of the more active tissues such as liver and intestine than to that of the less active tissues such as muscle or brain. A uniform phospholipid activity<sup>2</sup> per gm. of tissue, however, was not found in the four types of tumors investigated. The mammary carcinoma and the lymphosarcoma showed the highest phospholipid turnover; in these, phospholipid activity was at least twice as great as that observed in Sarcoma 180 and lymphoma.

Two explanations may be offered for the differences observed in the phospholipid activity of these tumors: (1) each tumor may have a characteristic phospholipid metabolism, or (2) the phospholipid activity of a tumor may be the result of effects produced upon the host by the tumor itself. In the latter case the

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<sup>1</sup> Jones, H. B., Chaikoff, I. L., and Lawrence, J. H., *J. Biol. Chem.*, **128**, 631 (1939).

<sup>2</sup> The term phospholipid activity refers to the percentage of administered labeled phosphorus found in the form of phospholipid. Throughout this study activity per gm. is recorded.

observed differences in phospholipid activity would merely reflect the alterations in the phosphorus metabolism of the host. In order to determine whether the phospholipid activity of a tumor is a primary characteristic of that tissue or whether it is secondary, *i.e.* induced by the host, a new type of tumor inoculation was devised, in which two or three separate varieties of neoplastic tissue were permitted to develop simultaneously in a single mouse. For the purpose of the present study different neoplasms growing side by side in the same animal are referred to as *fraternal tumors*.

#### EXPERIMENTAL

The number of animals used and their treatment are summarized in Table I. Mice of the A strain were used throughout. Tumors were produced by inoculation of tumor particles by the trochar method. Each animal received transplants of two or three types of neoplastic tissues. In the case of double inoculations, both axillary regions were employed. In the animals in which three types of tumors were permitted to develop simultaneously, inoculations were made in the nape in addition to both axillae.

Double inoculations of the following combinations of fraternal tumors were effectively grown in the same mouse: (1) mammary carcinoma and lymphosarcoma, (2) lymphoma and lymphosarcoma, (3) mammary carcinoma and lymphoma. Sarcoma 180, which was included in the previous report, was not investigated, since it takes poorly in the A strain of mouse. In the first three groups of animals recorded in Table I, double inoculations were employed. 10 days after transplantation, each tumor appeared as a small nodule weighing less than 0.1 gm. The growth curves of the tumors grown under the present conditions of double inoculation did not differ from those previously observed when each tumor was grown separately.<sup>1</sup> Phospholipid activities of these neoplastic tissues were determined at an early age, 11 to 15 days after inoculation. When excised, the tumors had attained weights of 0.08 to 0.62 gm. and were present as solid masses of tissue that showed neither necrosis nor cystic degenerations. At this early age, evidence of metastases such as enlarged lymph nodes and spleen was not found.

In a single group of animals, triple inoculations were employed.

Tumors growing under such conditions appear at 10 days as small nodules weighing less than 0.1 gm. Observations on a group of sixteen mice with triple transplants were made for as long as 16 days after inoculation and during this time their growth and development resembled those found when each tumor was grown

TABLE I  
*Summary of Tumor Experiments*

Group and Fig. No.	No. of mice used*	Weight of mice†		0.60 mg. P as Na <sub>2</sub> HPO <sub>4</sub> injected per mouse		Tumors inoculated	Symbol in chart	Tumor			
		Range	Average	Volume	Microcuriest			Initial sample		Final sample	
								Age	Average weight	Age	Average weight
		gm.	gm.	ml.				days	gm.	days	gm.
1	36	20-23	21	0.20	6	Mammary carcinoma	●	11	0.08	13	0.21
						Lymphosarcoma	○	11	0.18	13	0.35
2§	22	18-23	21	0.20	5	"	○	13	0.27	15	0.48
						Lymphoma	○	13	0.60	15	0.62
3§	30	21-27	23	0.20	5	Mammary carcinoma	●	11	0.15	15	0.36
						Lymphoma	○	11	0.33	15	0.60
4	4	27-29	28	0.20	2	Mammary carcinoma	●	25	0.62	26	0.63
						Lymphosarcoma	○	25	0.72	26	0.70
						Lymphoma	○	25	0.55	26	0.55

\* Mice approximately 3 months of age and of both sexes were used. The phospholipid activities recorded were apparently independent of the sex of the host.

† At the time of  $\text{P}^{32}$  administration.

‡ Activities standardized against uranium.

§ Gross evidence of metastases was not found.

|| Metastases had developed as shown by enlarged spleen and lymph nodes.

separately.<sup>1</sup> The group of mice recorded in Fig. 4, however, suffered from intestinal disturbances soon after being inoculated with tumor transplants, and in these mice the development of the tumors was somewhat retarded. The tumors did not reach a visible size until 15 to 17 days. But all animals used made a

complete recovery and the tumors continued to develop, attaining weights of 0.55 to 0.72 gm. 25 days after inoculation. Enlarged lymph nodes and spleen (*i.e.* evidence that the lymphoma was metastasizing) were found in these animals.

Each mouse received *intraperitoneally* 0.2 cc. of an isotonic solution of radioactive  $\text{Na}_2\text{HPO}_4$  containing 3 mg. of phosphorus per cc. The animals were sacrificed at various intervals thereafter and the tumors removed for analysis. The treatment of the neoplastic tissues has been recorded elsewhere. For the extraction of lipids from tumors weighing less than 0.5 gm. specially adapted Soxhlet apparatus of thimble size were used. The manner in which the phospholipids were isolated and their radioactivity determined has been described previously.<sup>1</sup> All values shown in Figs. 1 to 3 represent averages of five or more separate analyses.

### Results

*Double Inoculations of Mammary Carcinoma and Lymphosarcoma*—The values shown in Fig. 1 were obtained under conditions in which both tumors were developing simultaneously in a single mouse, the former in the right side of the animal and the lymphosarcoma in the left. In the previous report<sup>1</sup> dealing with the phospholipid metabolism of these neoplasms, bilateral inoculations of a single tumor type were employed. Thus, in both studies approximately the same mass of neoplastic tissue was exposed to 0.6 mg. of labeled phosphorus.

The phospholipid activities of mammary carcinoma and lymphosarcoma are in very close agreement in animals in which these tumors are growing side by side (Fig. 1). This is shown not only by the maximum values attained in relation to the time after the administration of radioactive phosphorus but also by the general shapes of the curves (Fig. 1). A close resemblance in phospholipid activities of these two tumors was also observed when they were grown separately in mice.<sup>1</sup>

In the case of single type inoculations,<sup>1</sup> mammary carcinoma attained its highest phospholipid activity about 10 hours after the injection of radioactive phosphorus; at this time about 1 per cent of the administered labeled phosphorus was deposited as phospholipid per gm. of tissue; at the 50 hour interval the amount of labeled phospholipid deposited was still not far from the maxi-

mum. These time relations were not strikingly altered when this tumor was grown beside lymphosarcoma in the same mouse (Fig. 1). Thus Fig. 1 shows maximum depositions of labeled phospholipid between 10 and 30 hours after the administration of radioactive phosphorus; at these times approximately 0.8 to 0.9 per cent of the administered labeled phosphorus was present

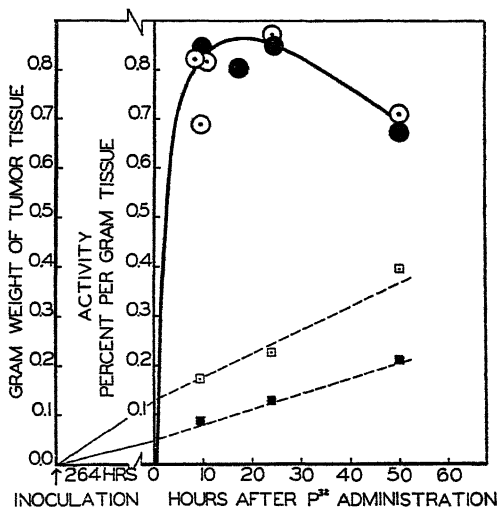


FIG. 1. The labeled phospholipid content per gm. of mammary carcinoma and lymphosarcoma in doubly inoculated mice. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of tissue. Each point is the mean of five or more separate determinations on as many mice. The broken lines are the growth curves for the tumors. ● phospholipid content of mammary carcinoma, ○ phospholipid content of lymphosarcoma, ■ weights of excised mammary carcinoma, □ weights of excised lymphosarcoma.

in each gm. of mammary carcinoma. Values close to the maximum were also found at the 50 hour interval.

The phospholipid activities of lymphosarcoma may be likewise compared. In general, similar activities were observed whether this type of tumor was permitted to develop in both axillary regions<sup>1</sup> or whether, as in the present study, it was confined to one side of an animal in which mammary carcinoma was growing in the other.

*Double Inoculations with Lymphosarcoma and Lymphoma*—In the previous study, in which a single type of tumor was permitted to invade each animal, it was found that lymphosarcoma was more than twice as active as lymphoma.<sup>1</sup> That the characteristic phospholipid metabolism of each tumor is retained when both are growing in the same animal is shown in Fig. 2. Phospholipid activities of both tumors in the same animal were studied at three

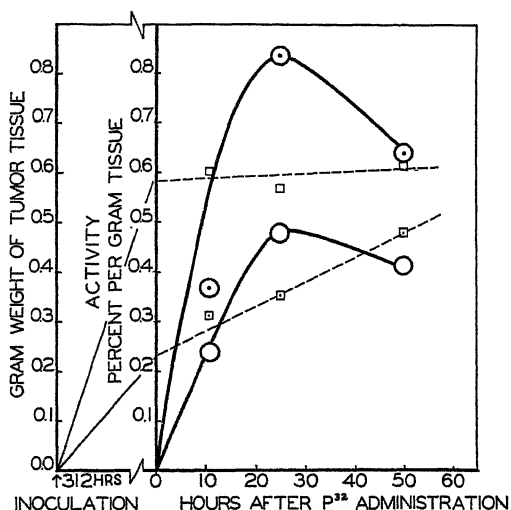


FIG. 2. The labeled phospholipid content per gm. of lymphosarcoma and lymphoma in doubly inoculated mice. The ordinates have the same meaning as for Fig. 1. Each point is the mean of five or more separate determinations on as many mice. The broken lines represent the growth of the tumors. ○ phospholipid content of lymphosarcoma, ○ phospholipid content of lymphoma, □ weights of excised lymphosarcoma, □ weights of lymphoma.

intervals after the injection of radioactive phosphorus, 11, 25, and 50 hours. Maximum activities for both tumors were found at the 25 hour interval, but at this time 0.83 and 0.47 per cent of the administered labeled phosphorus was present in the form of phospholipid in each gm. of lymphosarcoma and lymphoma respectively.

*Double Inoculations with Mammary Carcinoma and Lymphoma*—Fig. 3 was obtained from tumors removed from thirty mice,

each of which contained a lymphoma in the right axillary region and a mammary carcinoma in the left axillary region. The content of labeled phospholipid was determined in these tumors at 12.5, 29, 49, and 99 hours after the administration of radioactive phosphorus. In these doubly inoculated animals, the rate of turnover of labeled phospholipid was approximately twice as great in the mammary carcinoma as in the lymphoma. Maximum

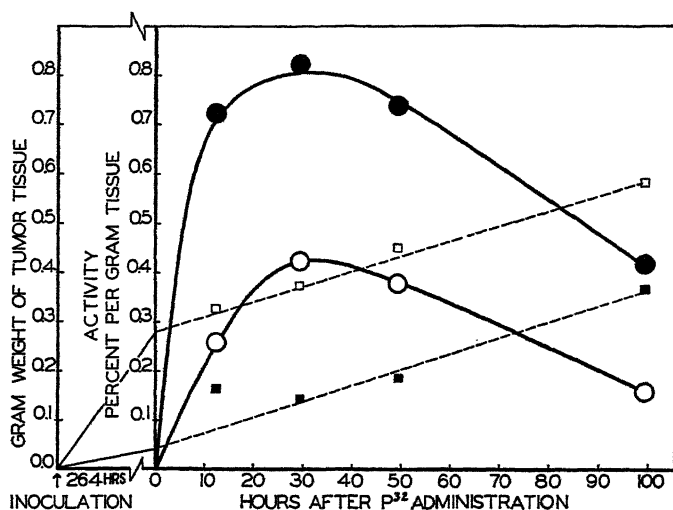


FIG. 3. The labeled phospholipid content per gm. of mammary carcinoma and lymphoma in doubly inoculated mice. The ordinates have the same meaning as for Fig. 1. Each point is the mean of five or more separate determinations on as many mice. The broken lines represent the growth of the tumors. ● phospholipid content of mammary carcinoma, ○ phospholipid content of lymphoma, ■ weights of excised mammary carcinoma, □ weights of excised lymphoma.

activities for both tumors were found between 10 and 50 hours after the administration of radioactive phosphorus, but at the highest point 0.82 per cent of the injected labeled phosphorus was contained in each gm. of mammary carcinoma, as compared with 0.43 per cent per gm. of lymphoma. At the 99 hour interval, mammary carcinoma contained 0.42 per cent of the radioactive phosphorus in the form of phospholipid and lymphoma 0.16 per cent per gm. of tissue. These values, observed under condi-



tions in which both tumors were present in the same animal, correspond closely with values previously found under conditions in which each neoplastic tissue was grown separately.<sup>1</sup> It is of further interest to note here that the phospholipid turnover of mammary carcinoma grown beside a lymphosarcoma (Fig. 1) is in good agreement with the turnover found for this same tumor grown beside a lymphoma (Fig. 3).

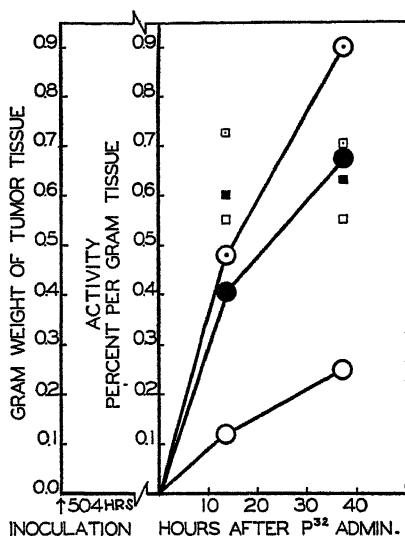


FIG. 4. The labeled phospholipid content per gm. of mammary carcinoma, lymphosarcoma, and lymphoma in triply inoculated mice. The ordinates have the same meaning as for Fig. 1. Each point is the mean of two separate determinations on as many mice. The symbols have the same meaning as those shown in Figs. 1 to 3.

*Triple Inoculations with Mammary Carcinoma, Lymphoma, and Lymphosarcoma*—Mice in which all three tumors had developed simultaneously were injected with  $\text{Na}_2\text{HPO}_4$  containing radioactive phosphorus, and their phospholipid activities determined at two intervals thereafter, 13 and 37 hours. Fig. 4 shows that the relative phospholipid activities observed above, when each animal contained two of these tumors, are roughly maintained under the present conditions, in which all three tumors had simultaneously

developed in the same host. Thus the lowest activities at both the early and the late hour were found for lymphoma. At the 37 hour interval, 0.25, 0.9, and 0.68 per cent of the administered labeled phosphorus was found in each gm. of lymphoma, lymphosarcoma, and mammary carcinoma respectively.

*Comment*—The present investigation focuses attention upon an interesting feature of neoplastic tissues. Each tumor maintains a characteristic phospholipid turnover whether grown singly or grown in the presence of two or three distinctly different types of tumors in the same animal. The fact that a lymphosarcoma, a mammary carcinoma, and a lymphoma failed to show identical rates of phospholipid turnover,<sup>3</sup> despite their exposure to the same host and hence to the same metabolic environment throughout their whole development, suggests that the phospholipid turnover of a tumor is not determined by the metabolism of the host. Although the individuality of the phospholipid metabolism of each type of tumor is thus established, it should nevertheless be noted that the possibility of the host's modifying the characteristic phospholipid turnover of a tumor is not ruled out.

#### SUMMARY

A method of tumor inoculation that permits the growth of two and three types of neoplastic tissues under identical metabolic environment has been employed for comparing the phospholipid activities of a mammary carcinoma, a lymphoma, and a lymphosarcoma. It is shown that each tumor possesses a characteristic phospholipid turnover which is apparently independent of the host.

<sup>3</sup> The turnover of total radioactive phosphorus has been measured and found to be quite similar in these three tumors.



## THE CONVERSION OF PALMITIC ACID INTO STEARIC AND PALMITOLEIC ACIDS IN RATS\*

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The properties of the depot fats of animals depend, to some extent, upon the nature of the dietary fats. Thus it was shown by Anderson and Mendel (1) that feeding of highly unsaturated oils results in the deposition of a body fat of high iodine number, whereas the administration of "hard" fat in the diet leads to a decrease in the iodine number of the depot fats. Recently Longenecker and Hilditch (2-4) have carefully reinvestigated this problem and they conclude that this change in the composition of the depot fat is due to the direct deposition of individual dietary fatty acids.

The effect of variation in diet upon the composition of the depot fat is limited, however, by the fact that all animals have a tendency to reproduce a depot fat with properties characteristic of their species. There are three types of biological reactions which may be responsible for this reproduction of the specific fatty acid mixture: (1) continuous synthesis and simultaneous destruction of fatty acids, (2) selective deposition of such fatty acids as are required and destruction of such dietary fatty acids as are present in excess or are not normal to the depot, (3) inter-conversion of one fatty acid into another.

In regard to the first of these mechanisms, the evidence for the

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formation of fatty acids from carbohydrates and proteins is too well known to require comment. Evidence for the continuous and unexpectedly rapid synthesis and destruction of depot fatty acids in normal animals on fat-free diets has recently been adduced, with the aid of deuterium (5). The second type of reaction, selective deposition, has been studied chiefly with respect to fatty acids of low molecular weight, which normally form only a minor component of the depots. It has been repeatedly shown (6) that fatty acids of 10 or less carbon atoms are not appreciably laid down in the depots, even when administered in large amounts in the diet.

The present paper deals mainly with the third mechanism; namely, the interconversions of fatty acids in the animal body. While there is some indirect evidence for the existence of such reactions, direct proof has been lacking. Such proof is not possible by any of the classical types of balance experiments, for the simultaneous interconversions of fatty acids will alter the composition of the depot fat only in so far as their rates are at variance. Measurements of variation in the amount of one or another fatty acid in the depots of animals on various diets are, at best, measurements of the difference between rate of appearance and rate of disappearance. As long as this type of evidence alone is adduced, neither the absolute rate of formation nor the precursor of the compound studied may be considered determined. Proof of the occurrence of such reactions may at present be obtained only if a "tracer atom," known to be stably attached originally to a molecule of one fatty acid, is recovered stably bound to a molecule of another fatty acid.

By this method it has recently been established that certain interconversions of fatty acids do occur in the animal body. By labeling stearic acid with deuterium, Schoenheimer and Rittenberg have been able to follow the fate of this compound in mice after its administration in the diet. In a series of papers (7-12) they have described the preparation of stearic acid containing deuterium, and the results of the feeding of its ethyl ester. Investigation of the deuterium content of various fractions of the depot fat of the animals thus fed showed that the bulk of the test substance was absorbed, that a portion was degraded, as evidenced by the presence of deuterium oxide in the body water, and

that another portion was stored as unchanged stearic acid in the depot. Part of the stearic acid was proved to have been degraded to palmitic acid (12), and part to have been desaturated (10). The reverse process, namely the hydrogenation of the double bonds in unsaturated fatty acids, was also shown to have occurred in the animal body (11).

In these earlier experiments, however, the diet contained considerable amounts of the test substance, and the possibility had to be considered that the conversion of the test substance into various products was an abnormal response to the enrichment of the diet. It was suggested, however, that the reactions that were observed to occur in the above experiments also took place in normal animals on normal diets (13).

In the earlier experiments (7), stearic acid was employed as a starting material because of the availability of unsaturated 18-carbon acids which could be reduced in an atmosphere of deuterium to the desired product. Recently, however, it has become possible to prepare the entire series of fatty acids labeled with deuterium by treating the corresponding normal fatty acid at about 130° with heavy water, platinum, and a trace of alkali, in a bomb tube (14). The deuterium thus introduced by exchange into the fatty acid molecule is "stably bound;" *i.e.*, it is not removed by any of the common reagents or reactions tried thus far, such as hot mineral acid, hot alkali, hydrogen-platinum catalyst at room temperature, sodium-ethanol reduction.

In the present experiment the test substance was deuteropalmitic acid containing 22.4 atom per cent deuterium. It was administered as the ethyl ester in a concentration of only 0.56 per cent of the diet. In order to insure an adequate supply of a variety of normal fatty acids, 6.4 per cent butter was also incorporated in the diet. From the quantity and deuterium content of the test substance, and from the amount of normal, non-isotopic palmitic acid known to occur in butter, it may be calculated that the mean deuterium content of the mixed dietary palmitic acid was 5.7 atom per cent.

Three growing male rats were used as test animals, growing rats being employed in preference to adult animals because of their ability to absorb and deposit fat rapidly. The diet was offered to them *ad libitum* for 8 days, and the animals were then

killed. The lipids isolated from the livers and from the remainder of the carcasses were fractionated by methods that have been previously described from this laboratory (10, 12).

*Introduction of Dietary Fatty Acids into Depot and Liver*—Of the deuterium fed as palmitic acid at least 92 per cent was found to have been absorbed and 44 per cent was recovered in the fatty acids of the depot fat. The high rate of deposition of dietary fatty acid is in close agreement with previous experiments in which fatty acids tagged with deuterium were fed to mice (9).

In the present experiment, the palmitic acid was administered as its ethyl ester. It is known, however, that dietary ethyl esters do not result in the deposition of any ethyl esters in the body fat (15). It is, therefore, necessary to assume either that new neutral fats were synthesized from the fatty acid of the diet, or that fatty acid molecules of the diet had replaced others already present in triglycerides in the depots.

In addition to the presence of deuterium in the depot fat, relatively high deuterium concentrations were found in the fatty acids of the liver. Both the saturated and unsaturated fatty acids of this organ were found to have deuterium concentrations about 2.5 times as great as the corresponding fractions of the depot fat. This is in accord with the findings of Barrett, Best, and Ridout (16), who also found, after feeding deuterio fats, that the liver fatty acids were richer in isotope than those of the rest of the carcass.

The total fatty acids of the carcass, containing 0.50 atom per cent deuterium, were fractionated, and several individual fatty acids were isolated (Table I). In procedures dealing with the conversion of an isotopic compound, A, into another compound, B, special precautions must be taken to insure that any isotope found in Compound B is not due to contamination with Compound A. As the various fatty acids are difficult to separate completely, and as traces of deuteropalmitic acid had to be considered as likely contaminants of each of the other fractions, "washing out" procedures, previously described from this laboratory (10, 12), were instituted wherever feasible.

*Deposition of Dietary Palmitic Acid*—As will be seen in Table I, the fatty acid of the carcasses that was richest in deuterium was palmitic acid. This was to be expected, and is in accord with the

previous finding (10, 12) that when deuterostearic acid was fed the carcass stearic acid contained a higher concentration of deuterium than any of the other fractions. If the deuterium content of the palmitic acid of the carcass be compared with that of the dietary palmitic acid, it will be found that  $1.38/5.7 = 0.24$ , or about 24 per cent of all the palmitic acid in the carcasses was derived from the direct deposition of dietary palmitic acid. Since, according to Longenecker (3) about 20 per cent of the 30 gm. of fatty acid isolated from the carcasses should have been palmitic acid, about 1.4 gm. of dietary palmitic acid were thus deposited.

TABLE I

*Deuterium Content of Fatty Acids Isolated from Carcass Fat of Rats Fed Deuteropalmitic Acid*

The mean deuterium content of dietary palmitic acid was 5.7 atom per cent.

Fatty acid	Deuterium
	<i>atom per cent</i>
Palmitic.....	1.38
Stearic.....	0.53
Mixture of myristic and lauric.....	0.32
Palmitoleic.....	0.36
Oleic.....	0.06
Linoleic.....	0.02

In addition to its occurrence in significant concentration in the carcass palmitic acid, deuterium was also found (Table I) in several other fatty acids.

*Conversion of Palmitic Acid into Stearic Acid*—The sample of stearic acid isolated from the depot contained 0.53 atom per cent deuterium, or about 38 per cent of that found in the carcass palmitic acid. This finding eliminates the possibility that the palmitic acid was first degraded to small fragments, such as acetaldehyde, and these subsequently recondensed to form an 18-carbon chain. In such a series of reactions, the bulk of the deuterium would have been lost, first, during the oxidative degradation, second, by exchange of deuterium with the normal hydrogen of the body water, and third, by dilution with the normal hydrogen required to reduce the double bonds that would result from aldol



condensations. The high deuterium content of the stearic acid must be taken as evidence for the direct elongation of the 16-carbon skeleton of palmitic acid to the 18-carbon chain of stearic acid, by the addition of 2 carbon atoms. Further experiments on this reaction and its biological mechanism will be presented in a subsequent paper.

*Degradation of Palmitic Acid to Acids of Lower Molecular Weight*—Part of the dietary palmitic acid was degraded to acids of lower molecular weight, chiefly myristic and possibly lauric acids. The deuterium content of this fraction (Table I) is 23 per cent of that found in the carcass palmitic acid.

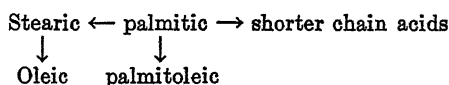
This finding is analogous to that described in an earlier experiment (12), in which, after the ingestion of large quantities of deuterostearic acid, deuteropalmitic acid was recovered from the carcass. It was taken as direct experimental confirmation of the theory of Knoop (17) and Dakin (18) that fatty acids are degraded by a process of one-sided  $\beta$  oxidation.

*Desaturation of Palmitic Acid to Palmitoleic Acid*—In 1906, an unnamed 16-carbon acid containing one double bond was isolated by Bull (19) from cod liver oil and herring oil. This was claimed by Grün (20) to be identical with the homologue of oleic acid described by Hofstädter (21) in 1854 as present in spermaceti. The compound was named palmitoleic acid by Lewkowitsch (22), and its structure proved by Toyama (23) to be  $\Delta_9,10$ -hexadecenoic acid. Banks, Hilditch, and Jones (24) first suspected, and Klenk, Ditt, and Diebold (25) definitely proved, its presence in the fat of rats. Longenecker (3, 4) has now found it to be a quantitatively important component of rat fat, comprising from 4 to 15 per cent of the total fatty acids of the carcass, depending upon diet.

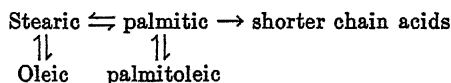
In a previous experiment, Schoenheimer and Rittenberg (10) had shown that deuterostearic acid is dehydrogenated by rats to oleic acid. In the present experiment, completely analogous findings indicate the desaturation of palmitic to palmitoleic acid in the body of the rat. From Table I it will be seen that the palmitoleic acid contained 26 per cent as much deuterium as the carcass palmitic acid. The low but significant concentration of deuterium in the oleic acid (Table I) is in accord with the previously published conversion of stearic to oleic acid.

*$\alpha$ -Linoleic Acid*—The fact that rats are unable to synthesize this compound was first suspected from observations (26) of the deficiency syndrome which resulted from its complete exclusion from the diet. Its disappearance from the depot fat of animals on such a diet has also been noted (27). The very low isotope content of the linoleic acid isolated in the present experiment (Table I) is in accord with these findings.<sup>1</sup>

*Interconversions of Fatty Acids Shown to Have Occurred*—The data presented in Table I are compatible with the following series of reactions.



Or, when these results are combined with those already obtained from previous isotope studies (10–12), the reactions may be formulated



In consideration of the transformations that the labeled palmitic acid underwent after ingestion, it should again be emphasized that each of the fatty acids into which the test substance was transformed was liberally supplied in the butter of the diet. In other words, *the rat can and does continuously convert palmitic acid into several other fatty acids, even though these products are available from other sources.*

*Estimates of Amounts of Various Fatty Acids Formed from Palmitic Acid*—The palmitic acid of the diet, comprising normal palmitic acid of butter together with deuteropalmitic acid added, contained 5.7 atom per cent deuterium. If this figure be compared with the analytical values in Table I, it may be computed that 24 per cent of the carcass palmitic acid, 9.3 per cent of the

<sup>1</sup> More recently, Bernhard and Schoenheimer (forthcoming publication) have shown that, whereas palmitic, stearic, and oleic acids of mice, whose body water was enriched with respect to deuterium oxide, contained considerable quantities of deuterium, the linoleic acid isolated from these animals contained no significant quantity of deuterium, indicating the absence of synthesis of this compound.

carcass stearic acid, and 6.3 per cent of the carcass palmitoleic acid were derived directly from the palmitic acid of the diet. This calculation presupposes, however, that the dietary palmitic acid is converted into other fatty acids prior to any dilution with the large reservoir of normal, "non-isotopic" palmitic acid already present in the depot fat. If the depot palmitic acid (1.38 atom per cent deuterium) be considered as the immediate precursor of the other fatty acids, it may be estimated that 38 per cent of the stearic acid and 26 per cent of the palmitoleic acid of the carcass were derived from this source.

The results of the present experiment, showing the existence of continuous interconversions of fatty acids, call to mind similar reactions observed to occur with the amino acids of the animal proteins (28). The fatty acids of the depots, like the amino acids of the body proteins, undergo continuous transformations, even when the animals are in a uniform state of nutrition.

#### EXPERIMENTAL

*Materials and Methods*—Deuteropalmitic acid was prepared as previously described (14) by the platinum-catalyzed exchange reaction between heavy water and pure palmitic acid. The ethyl ester of this material contained  $19.9 \pm 0.3$  atom per cent deuterium corresponding to 22.4 atom per cent in palmitic acid. The ester melted sharply at  $24^{\circ}$ .

The basal diet contained 65 per cent corn-starch, 22.5 per cent casein, 6.25 per cent salt mixture (29), and 6.25 per cent yeast powder. 300 gm. of final diet were prepared by intimately mixing 279 gm. of the above basal diet with 19.31 gm. of butter and 1.69 gm. of ethyl deuteropalmitate. The mixture was offered freely to each of three growing male rats, weighing 132 to 150 gm. each, until they had consumed practically all of it. This required 8 days, and in that interval the rats showed an average gain in weight of 35 gm.

All deuterium analyses of the water obtained by combustion were performed by the falling drop technique (30). When substances rich in bromine had to be analyzed, the material was mixed with 10 times its weight of freshly precipitated silver, the water resulting from such a combustion containing but a trace of bromine. This could readily be removed by exposing the water to a small piece of bright copper-foil.

Equivalent weights of fatty acids were determined by titrating samples weighing about 75 mg. with 0.06 N alkali, with phenolphthalein as an indicator. Titration was so arranged that at the end-point the solvent was 1:1 ethanol-water. The alkali was standardized against samples of pure palmitic acid.

*Preliminary Separation of Lipids*—After the bulk of the diet was consumed, the rats were killed by a blow on the head. The gastrointestinal tracts were removed, from esophagus to anus, and combined with the feces and uneaten food for fat analysis. A sample of body water was distilled from the carcasses and found to contain  $0.03 \pm 0.02$  atom per cent deuterium. The carcasses and livers were separately investigated for fatty acids. Each fraction was hydrolyzed by refluxing for 2 hours with an excess of 10 per cent ethanolic KOH. Each reaction mixture was filtered through glass wool, the filtrate diluted with water, and the unsaponifiable material extracted from the alkaline solution with petroleum ether. The aqueous alcoholic solution was then acidified and the fatty acids extracted with petroleum ether. Each petroleum ether solution was washed with water, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated to dryness. The fatty acids of the gastrointestinal tracts, feces, and uneaten food (2.057 gm.) contained  $1.32 \pm 0.04$  atom per cent deuterium, and those of the carcasses (29.84 gm.)  $0.50 \pm 0.02$  atom per cent deuterium. 0.616 gm. of fatty acids was obtained from the livers. These were not analyzed, but were first separated into saturated and unsaturated fractions.

*Investigation of Carcass Fatty Acids*—29.0 gm. of carcass fatty acids were first separated into saturated ("solid") and unsaturated ("liquid") fractions by Twitchell's (31) lead salt method, as modified by Schoenheimer and Rittenberg (10). The precipitated lead soaps were recrystallized from hot ethanol and decomposed with strong HCl to give 5.93 gm. of saturated fatty acids containing  $1.04 \pm 0.03$  atom per cent deuterium. The unsaturated fatty acids, recovered from the soluble lead salts by treatment with  $\text{H}_2\text{S}$ , weighed 16.01 gm., and contained  $0.20 \pm 0.02$  atom per cent deuterium.

*Isolation of Palmitic Acid*—5.76 gm. of saturated fatty acids were converted into methyl esters and distilled in the modified micro-Widmer still with an electrically heated column that has been previously used in this laboratory (12). The details of the

TABLE II  
*Preliminary Distillation of Methyl Esters of Carcass Saturated Fatty Acids*

Pressure = 0.05 to 0.06 mm.

Fraction No.	Column temperature	Weight	M.p.	Remarks
	°C.	gm.	°C.	
1	118-134	0.619	18 -20	Low boiling portion, Table V
2	134-135	0.614	27 -28	} Combined for isolation of palmitic acid, Table III
3	133	0.509	27.5-28	
4	133	0.580	27	
5	133	0.589	27	
6	132-138	0.542	29 -30	
7	136-140	0.512	27.5-28.5	
8	140-155	0.703	22 -25	
9	153-154	0.443	32 -33	} Combined for isolation of stearic acid, Table IV
10	155-174	0.260	34 -35	
11	Residue	0.289	34 -35	

TABLE III  
*Purification of Carcass Palmitic Acid*

Methyl esters, Fractions 2 to 7, Table II, combined and distilled. Pressure = 0.07 mm.

Fraction No.	Methyl esters		
	Column temperature	Weight	M.p.
	°C.	gm.	°C.
1	137-138	0.509	27-28
2*	138-139	0.578	29
3*	138-142	0.542	29
4*	135-141	0.400	29
5	137 up		28

\* Fractions 2, 3, and 4 were combined and saponified. The liberated fatty acid, after recrystallization, melted at 62.0°, had an equivalent weight of 257.9, and contained  $1.38 \pm 0.03$  atom per cent deuterium.

distillation are given in Table II. Fractions 2 to 7 of this distillation, recombined and redistilled (Table III), yielded pure

methyl palmitate,<sup>2</sup> which, upon saponification, and recrystallization of the liberated acid from aqueous acetone, gave palmitic acid which contained  $1.38 \pm 0.03$  atom per cent deuterium.

*Isolation of Stearic Acid*—Fractions 9, 10, and 11 of the initial distillation (Table II) were combined, and twice "washed out" as previously described (12) by adding normal methyl palmitate and distilling it off. It will be seen from Table IV that only

TABLE IV

*Purification of Carcass Stearic Acid by "Washing Out" Procedure*

Methyl esters, Fractions 9 to 11, Table II, + 1.00 gm. of normal methyl palmitate combined and distilled. Pressure = 0.06 mm.

Fraction No.	Methyl esters			
	Column temperature	Weight	M.p.	Deuterium
	°C.	gm.	°C.	atom per cent
1	135-137	0.100		
2	136-138	0.644		
3	139-146	0.239		0.12 $\pm$ 0.02
0.50 gm. normal methyl palmitate added to residue in still, and distillation continued				
4	134-135	0.181		
5	137-139	0.155		0.05 $\pm$ 0.02
6	139-153	0.236	23-25	
7	154	0.077	31-32	0.44 $\pm$ 0.02
8*	154-170	0.469	34-35	
9	Residue			0.20 $\pm$ 0.02

\* Fraction 8 was saponified. The liberated fatty acid, after recrystallization, melted at 65.4°, had an equivalent weight of 284.3, and contained  $0.53 \pm 0.02$  atom per cent deuterium.

after the deuterium content of the contaminating methyl palmitate had been reduced to 0.05 atom per cent was the methyl stearate distilled. The stearic acid derived from this material contained  $0.53 \pm 0.02$  atom per cent deuterium.

<sup>2</sup> The purity of fractions of these distillations may be inferred from the melting point and equivalent weight data. The theoretical equivalent weight of palmitic acid is 256.3, of stearic acid, 284.3. Melting points that have been obtained in this laboratory are methyl palmitate 29.5°, methyl stearate 38°, palmitic acid 62.4°, stearic acid 69°.

*Isolation of Shorter Chain Fatty Acids*—In like fashion, the low boiling saturated fatty acid methyl esters (Fraction 1, Table II) were twice "washed out" by distilling them away from portions of normal methyl palmitate twice in succession, as indicated in Table V. By this means the deuterium concentration of the contaminating methyl palmitate was reduced to  $0.11 \pm 0.02$  atom per cent. The low boiling methyl esters were found to contain  $0.29 \pm 0.02$  atom per cent deuterium, which, calculated on the basis of free myristic acid, would give a deuterium concen-

TABLE V

*Purification of Low Boiling Saturated Fatty Acid Methyl Esters from Carcasses, by "Washing Out" Procedure*

Methyl esters, Fraction 1, Table II, + 1.00 gm. of normal methyl palmitate combined and distilled. Pressure = 0.05 to 0.06 mm.

Fraction No.	Methyl esters			
	Column temperature	Weight	M.p.	Deuterium
	°C.	gm.	°C.	atom per cent
1	121-130	0.469		
2	133-135			
3	133			$0.43 \pm 0.02$
Still cleaned out, and Fraction 1, above, + 1.00 gm. normal methyl palmitate combined and distilled				
4	117-130	0.359	22-24.5	$0.29 \pm 0.02$
5	131-133	0.313	22-25	$0.12 \pm 0.02$
6	131-135	0.254	29	$0.11 \pm 0.02$
7	Residue			$0.09 \pm 0.02$

tration of  $0.32 \pm 0.02$  atom per cent. The fraction was too small to permit of isolation of pure individual fatty acids.

*Investigation of Unsaturated Fatty Acids from Carcass*—The unsaturated fraction of carcass fatty acids obtained from the initial lead soap separation was found to contain  $0.20 \pm 0.02$  atom per cent deuterium. 14.33 gm. of this material were "washed out" as previously described (10) by mixing with 1 gm. each of normal palmitic and stearic acids and precipitating with lead acetate. After filtering, the filtrate was heated to boiling and a second portion of palmitic and stearic acids, followed by 2 gm. of lead

acetate, was added. The precipitate was again removed and the operation repeated a third time. Each precipitate of lead soap was recrystallized from ethanol, and the derived saturated fatty acid mixture analyzed for deuterium. After the third "washing out," the precipitate contained only  $0.05 \pm 0.02$  atom per cent deuterium (Table VI). The unsaturated fatty acids were now regenerated by  $H_2S$  from the filtrate of the last lead precipitation and gave 8.94 gm. of a pale yellow oil which contained  $0.15 \pm 0.02$  atom per cent deuterium.

*Isolation of Linoleic Acid*—Linoleic acid was isolated from the above material as the tetrabromide, according to Rollett (32), by

TABLE VI  
*Purification of Unsaturated Fatty Acids of Carcass by  
"Washing Out" Procedure*

Unsaturated fatty acids were mixed three times in succession with normal palmitic and stearic acids and separated by the lead precipitation method.

Fraction	Deuterium
	<i>atom per cent</i>
Saturated fatty acids of carcass.....	$1.04 \pm 0.02$
Precipitate from 1st "washing".....	$0.29 \pm 0.02$
"        "        2nd        ".....	$0.09 \pm 0.02$
"        "        3rd        ".....	$0.05 \pm 0.02$
Unsaturated fatty acids of carcass.....	$0.15 \pm 0.02$

adding bromine to the oil in petroleum ether solution at  $0^\circ$  up to the persistence of a brown color. The resultant precipitate was filtered off, washed well with cold petroleum ether, and dried. The pale gray powder, melting at  $112^\circ$ , contained 54.7 per cent bromine by micro-Carius determination (theory for tetrabromostearic acid, 53.3 per cent, for hexabromostearic acid, 63.1 per cent). The water derived from its combustion contained  $0.02 \pm 0.02$  atom per cent deuterium.

*Isolation of Oleic and Palmitoleic Acids*—Oleic and palmitoleic acids were not isolated as such but were first reduced and the corresponding saturated stearic and palmitic acids separated. The mother liquor from the above bromine precipitation, comprising chiefly bromo derivatives of oleic, palmitoleic, and traces of



linoleic acids, was evaporated to dryness and the residue debrominated by refluxing with an excess of zinc dust in glacial acetic acid. The resultant debrominated fatty acids were completely reduced by shaking with normal hydrogen in the presence of active platinum catalyst at room temperature and atmospheric pressure.

TABLE VII

*Distillation of Saturated Fatty Acid Methyl Esters Prepared by Reduction of Oleic Acid, Palmitoleic Acid, Etc., from Carcasses of Rats Fed Ethyl Deuteropalmitate*

Pressure = 0.05 to 0.06 mm.

Fraction No.	Methyl esters		
	Column temperature	Weight	M.p.
	°C.	gm.	°C.
1	100-131	0.419	26
2	129-131	0.130	
3	132-135	0.590	
4	135-142	0.508	
5	148-149	0.535	37.5-38
6*	148-153	0.646	
Fraction 3 + 4, recombined and redistilled			
1a†	127-129	0.120	29
2a†	125-128	0.145	29
3a	128-129	0.196	25 -25.5
4a	129-134	0.127	29 -32

\* Fraction 6 was saponified. The derived fatty acid, after recrystallization, melted at 68.4°, had an equivalent weight of 282.0, and contained 0.06 ± 0.02 atom per cent deuterium.

† Fractions 1a and 2a were combined and saponified. The derived fatty acid, after recrystallization, melted at 62.4°, had an equivalent weight of 256.7, and contained 0.34 ± 0.02 atom per cent deuterium.

Under these conditions, no significant exchange of carbon-bound hydrogen in fatty acids is to be expected (14). 7.04 gm. of fatty acid took up 450 cc. of hydrogen before the reaction was completed. The saturated fatty acids were now converted to methyl esters in the usual fashion, and the mixed methyl esters fractionally distilled. The data of this distillation are given in Table VII. It will be seen that the sample of palmitic acid obtained contained

$0.34 \pm 0.02$  atom per cent deuterium. This corresponds to  $0.36 \pm 0.02$  atom per cent deuterium in the palmitoleic acid from which it was made. The stearic acid obtained from the distillation contained  $0.06 \pm 0.02$  atom per cent deuterium, which corresponds to  $0.06 \pm 0.02$  atom per cent in the oleic acid from which it was derived.

*Investigation of Fatty Acids from Livers*—0.616 gm. of liver fatty acids was separated into saturated and unsaturated fractions by the lead soap procedure. The soluble lead soaps were once "washed out" by adding to the hot ethanolic solution 50 mg. each of normal palmitic and stearic acids followed by 100 mg. of lead acetate. The second crop of precipitate which formed was filtered off. The two portions of precipitated lead salt were recrystallized from hot ethanol and decomposed in the usual fashion with HCl. The soluble lead salts were decomposed with  $H_2S$ . The saturated fatty acids of the livers contained  $2.49 \pm 0.03$  atom per cent deuterium, and the unsaturated fraction contained  $0.42 \pm 0.02$  atom per cent. The saturated fatty acids recovered from the "washing out" contained  $0.41 \pm 0.03$  atom per cent deuterium. The deuterium concentrations in the two fractions of liver fatty acids parallel the corresponding values in the carcass fatty acids, each fraction of liver fatty acid containing about 2.5 times the concentration of isotope found in the analogous fraction of carcass fatty acid.

#### SUMMARY

1. Deuteropalmitic acid in the form of its ethyl ester was added as a quantitatively minor component to a diet otherwise generously supplied with a variety of normal fatty acids, and this diet was fed to rats. After 8 days, the animals were killed, and various fractions of body fat, including several individual carcass fatty acids, were isolated and analyzed for deuterium.

2. The bulk of the test substance was absorbed, and about 44 per cent of the deuterium fed as palmitic acid was recovered from the fat of the animals. Much of the deuteropalmitic acid had been deposited directly in the tissues.

3. Part of the palmitic acid was degraded to acids of shorter chain length; another part was desaturated to palmitoleic acid. A novel reaction was the finding of the conversion of palmitic acid

into stearic acid, representing a direct elongation of the carbon chain of palmitic acid by 2 carbon atoms.

4. The new fatty acids were formed despite the fact that the animals were ingesting, during the course of the experiment, an abundance of the same fatty acids, supplied as butter in the diet.

5. These conclusions are coordinated with the previous reports of studies in the metabolism of fatty acids, and a scheme is proposed to indicate certain interconversions of fatty acids that are normally and continuously taking place in the animal body.

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## THE BIOLOGICAL RELATIONS OF THE HIGHER ALIPHATIC ALCOHOLS TO FATTY ACIDS\*

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Almost 50 years ago, Munk and Rosenstein (1) reported the feeding of cetyl palmitate, as spermaceti, to a patient suffering from a chronic chyle fistula. Whereas they could recover a good portion of the palmitic acid, as tripalmitin, from the chyle, they were unable to demonstrate the presence of any cetyl alcohol in this material. It has since been shown by Mancke (2) that, even when large amounts of cetyl alcohol were added to the diet, the rise in cetyl alcohol excretion in the feces accounted for but a small fraction of that fed. Even after intensive feeding, however, he was unable to demonstrate any cetyl alcohol in the depot fat, the milk, or the lymph of his experimental animals. He concluded that the alcohol was rapidly destroyed by the animal. Gardner (3) discovered cetyl alcohol to be a normal but minor constituent of mammalian feces, and this finding has been confirmed (4).

Schoenheimer and Hilgetag (4) were able to isolate cetyl alcohol, and on occasion small amounts of octadecyl alcohol, not only from normal mammalian feces, but also from the sterile feces of new born infants, the intestinal wall, the feces of dogs with biliary fistulae, and the contents of sterile operative intestinal cysts. Having thus successively eliminated bacterial action, diet, and bile as sources of the cetyl alcohol found, they concluded that

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it was a secretory product of the intestinal mucosa. Furthermore, in view of the extreme readiness with which ingested cetyl alcohol is absorbed from the gastrointestinal tract, the fact that this compound occurs in the feces at all shows it to be a normal metabolic intermediary, for a great deal of it would have to be secreted into the lumen of the gut for a small quantity to escape the efficient absorption mechanism.

*Conversion of Palmitic Acid into Higher Alcohols*—In the present work an attempt was made to determine whether the naturally occurring fatty acids act as the biological precursors of the higher aliphatic alcohols normally excreted in the feces. In a previous paper (5) an experiment was reported in which rats were fed for 8 days with deuteropalmitic acid, and its conversion into other fatty acids was studied. We have now investigated the unsaponifiable matter obtained from the feces of these animals. The sterols were first quantitatively removed as digitonides and found to contain no excess of isotope. The fraction volatile below 80° under 0.02 mm. pressure was freed of non-alcoholic contaminants via the sodium salts of the succinic hemiesters. Only 22 mg. of hemisuccinates were obtained, an amount too small for further purification. This material contained 0.72 atom per cent deuterium, calculated on the basis of cetyl alcohol, which is about half the concentration of isotope that had been found in the palmitic acid obtained from the bodies of these animals (1.38 atom per cent).

It should be pointed out that the feces analyzed had been collected from the time of initiation of feeding of the isotopic test substance up to the conclusion of the experiment. Any alcohols excreted during the first few days of feeding must have had a lower isotope content than those excreted later on. Thus, the alcohols excreted on the last day of feeding, which would be strictly comparable with the carcass palmitic acid, would necessarily have a higher deuterium content than the accumulated material which was actually analyzed.

In view of the fact that cetyl alcohol is the only compound that has been isolated with any consistency (4) from the fraction studied, it is reasonable to suppose that it was a major component of the material analyzed for deuterium. The experimental finding points strongly to the conversion of palmitic acid into alcohols of high molecular weight, and is taken as indication of the conversion specifically to cetyl alcohol. As the biological conversion observed

in this experiment was investigated in normal animals, its occurrence must be a normal event.

*Conversion of Higher Alcohols into Fatty Acids*—Experiments were carried out to investigate whether the reverse of the reaction described above, namely the conversion of higher alcohols into fatty acids, also occurs in the animal body. Deuterium-containing cetyl and octadecyl alcohols were synthesized in the laboratory and their acetates administered to rats as 1 per cent of an otherwise normal stock diet, containing 6 per cent butter, for a period of 8 to 9 days. Both test substances were excellently absorbed, a finding in accord with all previous investigations. The unsaponifiable fractions of the hydrolysates of the bodies of these animals must have contained any higher alcohols that were present. The deuterium content of these fractions accounted for only traces of unchanged cetyl and octadecyl alcohols. The fatty acids of the carcasses, however, accounted for 36 to 47 per cent of the dietary isotope bound to alcohol. The remainder of the test substance was probably degraded.

The saturated fatty acids of the bodies of these animals were fractionated according to methods previously described (5). When deuterocetyl alcohol was fed, the corresponding palmitic acid of the carcass showed a high content of deuterium, while the isotope content of the stearic acid was lower. Administration of deuteriooctadecyl alcohol resulted in a very high deuterium content in the carcass stearic acid and a considerably lower value for the palmitic acid.

The findings in these experiments considered together indicate that normal animals continuously convert not only fatty acids into alcohols, but, conversely, higher aliphatic alcohols into fatty acids. The quantitative data obtained in the deuterium analyses of fatty acids after feeding deuterocetyl alcohol are remarkably similar to those obtained after feeding deuteropalmitic acid (5). It seems that the conversion of cetyl alcohol to palmitic acid is so rapid that it makes little difference whether the deuterium is administered bound to palmitic acid or to cetyl alcohol. The rapidity of this oxidation doubtless explains the inability of earlier workers (1, 2) to recover cetyl alcohol after its feeding.

In the previous paper (5) it was shown that normal rats elongate the chain of palmitic acid to form stearic acid. In view of the relative chemical inertia of carboxyl groups as reactants in con-



condensations of this sort, a more reactive derivative of palmitic acid must be considered to occur as a biological intermediate. The high biological activity of cetyl alcohol suggested that this substance, or, what is more likely, the corresponding aldehyde,<sup>1</sup> was the required reactant in this condensation.

In order to test the effectiveness of cetyl alcohol as a precursor of stearic acid in the animal body, a third feeding experiment was designed in which the butter of the earlier diet was replaced by normal, non-isotopic ethyl palmitate. Deuterocetyl alcohol, as its acetate, comprised 2 per cent of the diet. As the diet was free of stearic acid, any of this acid deposited must have been formed by synthesis. At the same time, the deuterium content of the depot palmitic acid, formed in part from deuterocetyl alcohol, would be reduced by the continuous deposition of non-isotopic dietary palmitic acid. At the conclusion of the feeding, carcass palmitic and stearic acids were isolated. The former contained 0.44, the latter, 0.34 atom per cent deuterium. In other words, the stearic acid had an isotope content about 77 per cent of that in the palmitic acid. If these results, as well as those of the previous experiment, be compared with those obtained after feeding deuteropalmitic acid (5), it may be concluded that cetyl alcohol is at least as effective a precursor of stearic acid as is palmitic acid. The results, while showing a high activity of cetyl alcohol in forming stearic acid, are not regarded as final proof, but the likelihood exists that the alcohol is formed from palmitaldehyde, either by the animal or in the course of isolation, and that the aldehyde is an intermediate in the conversion of palmitic to stearic acid.

#### EXPERIMENTAL

*Preparation of Deuterocetyl and Deuterooctadecyl Alcohols—*  
An attempt to prepare deuterocetyl alcohol by exchange reaction

<sup>1</sup> Popular speculation (6, 7) on the biological synthesis of fatty acids emphasizes the likelihood of "aldol" types of condensation reactions. Since palmitaldehyde has been shown by Feulgen and collaborators (8, 9) to be a normal constituent of animal tissues, and since this aldehyde must be considered as an intermediate in the cetyl alcohol-palmitic acid oxidation-reduction system, it is possible that this substance occupies a central position between palmitic acid, cetyl alcohol, and stearic acid. Study of its metabolic relations will be of interest.

between cetyl alcohol and heavy water under conditions (10) which had been employed in the preparation of deuterio fatty acids was unsuccessful, the product containing only traces of deuterium. The alcohols were therefore prepared from the corresponding deuterio acids. Deuteropalmitic acid was prepared by exchange reaction with heavy water (10) and deuterostearic acid by the platinum-catalyzed reduction of oleic acid in an atmosphere of deuterium (11). These acids were esterified with ethyl alcohol in the usual fashion, and the ethyl esters reduced to alcohols with sodium "sand" and ethanol (12). The alcoholic products were acetylated by refluxing with acetic anhydride and the alcohol acetates purified by distillation. Deuterocetyl acetate boiled at 180–194° under 15 to 16 mm. of mercury pressure. It melted at 24°, and proved to contain  $11.2 \pm 0.4$  atom per cent deuterium, corresponding to 11.9 atom per cent in the free alcohol. Deuterooctadecyl acetate distilled under a pressure of 4 mm. of mercury at 155–161°. The distillate melted at 32° and contained  $5.4 \pm 0.2$  atom per cent deuterium, corresponding to 5.7 atom per cent in the octadecyl alcohol. The melting points obtained agree well with those of the  $\beta$  modification of the crystal structure of the alcohol acetates, according to Meyer and Reid (13).

*Feeding of Deuterooctadecyl Alcohol*—93 gm. of the basal diet previously described (5) were intimately mixed with 6 gm. of butter and 1 gm. of deuterooctadecyl acetate. The mixture was offered *ad libitum* to an immature male rat weighing 111 gm. After 9 days the animal had consumed the major part of the diet and had gained 25 gm. in weight. Neither during this nor during subsequent experiments was any evidence of the supposed purgative action of cetyl and octadecyl alcohols noted (14).

The animal was killed by a blow on the head and the lipids fractionated as described in the previous paper (5). The weights and deuterium content of the various fractions of this initial separation are given in Table I.

The carcass fatty acids were divided into saturated and unsaturated fractions by the lead salt method (15, 16), and the free saturated fatty acids, recovered from the recrystallized insoluble lead soaps, esterified with methyl alcohol. 1.35 gm. of saturated fatty acid methyl esters were obtained and this material frac-

tionally distilled in a micro-Widmer apparatus previously used in this laboratory (17). The details of this distillation are given in

TABLE I  
*Initial Fractionation of Lipids of Rats Fed Deutero Alcohol Acetates*

Fraction	Rat 1, fed deuterooctadecyl acetate		Rat 2, fed deuterooctyl acetate	
	Weight	Deuterium	Weight	D uterium
	gm.	atom per cent	gm.	atom per cent
Body water.....		0.03 $\pm$ 0.02		0.04 $\pm$ 0.02
Feces, uneaten food, and gastrointestinal tracts. Total lipids.	0.778	0.54 $\pm$ 0.02	0.667	0.54 $\pm$ 0.02
Livers. Fatty acids...	0.153	0.78 $\pm$ 0.02	0.248	0.65 $\pm$ 0.02
“ Unsaponifiable.....	0.014	0.63 $\pm$ 0.10	0.011	0.94 $\pm$ 0.20
Carcasses. Fatty acids.....	9.773	0.26 $\pm$ 0.02	11.880	0.26 $\pm$ 0.02
Carcasses. Unsaponifiable.....	0.287	0.10 $\pm$ 0.02	0.440	0.26 $\pm$ 0.03

TABLE II  
*Distillation of Methyl Esters of Saturated Fatty Acids from Carcass of Rat Fed Deuterooctadecyl Acetate*

Pressure = 0.1 mm.

Fraction No.	Methyl esters			Free acids		
	Column temperature	Weight	M.p.	M.p.	Equivalent weight	Deuterium
	°C.	gm.	°C.	°C.		atom per cent
1	119-131	0.052	<15			0.06 $\pm$ 0.03
2	132-136	0.155	22-25	58.2		0.08 $\pm$ 0.02
3	135-136	0.250	27-29	61.3	254.3	0.10 $\pm$ 0.02
4	137-138	0.183	29	61.8	255.1	0.13 $\pm$ 0.03
5	137-139	0.167	29-29.5	61.6	256.6	0.10 $\pm$ 0.02
6	142-145	0.158	29	60.6	254.2	0.11 $\pm$ 0.02
7	142-157	0.132	18-22	54.0		0.65 $\pm$ 0.04
8	170-174	0.163	33-35	65.8	280.1	1.15 $\pm$ 0.05

Table II. The composition of the various fractions may be estimated from melting points of the esters, as well as the equivalent weight and melting point values of the recrystallized derived

fatty acids. Fractions 3 to 6 all represent fairly pure palmitic acid which will be seen to contain 0.11 atom per cent deuterium. Fraction 8 is a mixture of about 80 per cent stearic acid and 20 per cent palmitic acid. From this it may be estimated that pure stearic acid must contain about 1.3 atom per cent deuterium. The difficulty encountered in this and subsequent distillations in obtaining relatively pure samples of stearic acid arises from the scant quantity of this acid in rat fat.

TABLE III

*Distillation of Methyl Esters of Saturated Fatty Acids from Carcass of Rat Fed Deuterocetyl Acetate*

Pressure = 0.08 mm.

Fraction No.	Methyl esters			Free acids		
	Column temperature	Weight	M.p.	M.p.	Equivalent weight	Deuterium
	°C.	gm.	°C.	°C.		atom per cent
1	118-126	0.180	<15			0.27 ± 0.02
2	128-131	0.165	29.5	61.8	258.3	0.59 ± 0.02
3	131-132	0.201	29.5	62.0	258.3	0.57 ± 0.02
4	130-131	0.253	29.5	61.7	254.8	0.58 ± 0.02
5	134-135	0.362	29	61.4	255.0	0.53 ± 0.02
6	134-140	0.187	29	62.0	257.2	0.51 ± 0.02
7	145-160	0.146	23-24			0.39 ± 0.02
8	164	0.151	32	64.6	280.8	0.33 ± 0.05
9	164-180	0.094	35.5	66.2	286.7	0.40 ± 0.04
10	Residue	0.120				0.22 ± 0.02

*Feeding of Deuterocetyl Alcohol*—The diet was identical with that of the previous experiment except that deuterocetyl acetate was the test substance instead of deuteriooctadecyl acetate. The animal in this case weighed 144 gm. and gained 26 gm. during the course of the feeding, which required 8 days. The same fractionation was carried out as before, and the data are given in Table I. Again the saturated fatty acids of the carcass were isolated and converted to methyl esters, in this case weighing 1.94 gm. The record of the distillation of this material is given in Table III. It will be seen that Fractions 2 to 4 represent palmitic acid of an equal purity which must contain about 0.58 atom per cent deuterium. Fraction 8 contains about 80 per cent stearic acid, Frac-

tion 9 about 85 per cent stearic acid, requiring that whatever the contaminant may be, pure stearic acid would have to contain at least some 0.30 atom per cent deuterium.

*Feeding of Deuterocetyl Alcohol together with Non-Isotopic Palmitic Acid*—The test substance in this experiment was the same deuterocetyl acetate that was used in the preceding experiment. 4 gm. of this material were intimately mixed with 186 gm. of the basal diet referred to above and 10 gm. of normal, non-isotopic ethyl palmitate. This diet was offered freely to three male rats weighing 160 to 200 gm. each. On the 6th day of feeding the bulk of the diet had been consumed and the animals had gained an average of 7 gm. The rats were killed at this time

TABLE IV  
*Initial Fractionation of Lipids from Rats Fed Deuterocetyl  
Acetate + Normal Ethyl Palmitate*

Fraction	Weight	Deuterium
	<i>gm.</i>	<i>atom per cent</i>
Body water.....		0.04 ± 0.02
Total fatty acids of bodies.....	39.2	0.26 ± 0.02
Saturated fraction.....	9.75	0.38 ± 0.02
Unsaturated fraction.....	18.02	0.17 ± 0.02
Unsaponifiable fraction of gastrointestinal tracts, feces, and uneaten food.....	0.753	6.2 ± 0.2

and worked up essentially as in the previous experiments, with the exception that the livers were not removed from the bodies. The weights and deuterium contents of the various fractions are given in Table IV.

9.5 gm. of saturated fatty acids of the carcasses were esterified with methanol and fractionally distilled. The results of this distillation are given in Table V. Two suitable fractions were selected for saponification, and the derived free acids recrystallized. Fraction 6 gave a melting point and an equivalent weight for a mixture of 95 per cent palmitic acid and 5 per cent stearic acid; Fraction 17 gave data for a mixture of 80 per cent stearic acid and 20 per cent palmitic acid. If the conventional assumption be made that in such a distillation no more than two members of a homologous series are found in each fraction (18), simultaneous

equations may be set up and solved. The deuterium content of pure palmitic acid proves to be 0.44, that of pure stearic acid, 0.34 atom per cent.

*Investigation of Alcoholic Fraction of Fecal Unsaponifiable Material of Rats Fed Deuteropalmitic Acid*—The material used in this

TABLE V

*Distillation of Methyl Esters of Carcass Saturated Fatty Acids from Rats Fed Deuterocetyl Acetate + Normal Ethyl Palmitate*

Pressure = 0.05 mm.

Fraction No.	Methyl esters		
	Column temperature	Weight	M.p.
	°C.	gm.	°C.
1	118-128	0.546	14 -18
2	128-132	0.602	27 -28
3	132	0.658	27.5-28
4	131-133	0.750	27.5-28.5
5	132	0.775	28 -29
6*	131-135	0.592	29
7	130-135	0.626	28.5-29
8	130-133	0.582	29
9	127-128	0.553	29 -30
10	128-130	0.675	29 -30
11	130-131	0.245	28
12	126-127	0.548	28 -29
13	130-136	0.691	23 -25
14	135-136	0.171	24 -27
15	133-140	0.653	24 -27
16	139-140	0.178	30 -32
17†	141-171	0.377	34 -35

\* Fraction 6 was saponified. The derived fatty acid, after recrystallization, melted at 61.2°, had an equivalent weight of 258.0, and contained  $0.44 \pm 0.02$  atom per cent deuterium.

† Fraction 17 was saponified. The derived fatty acid, after recrystallization, melted at 64.6°, had an equivalent weight of 279.9, and contained  $0.36 \pm 0.02$  atom per cent deuterium.

study was the unsaponifiable fraction of the hydrolysate of the combined gastrointestinal tracts, uneaten food, and feces obtained from three rats described in the previous paper (5). These rats had been fed for 8 days on a stock diet to which had been added 0.56 per cent ethyl deuteropalmitate, the palmitic acid of which

contained 22.4 atom per cent deuterium. 0.389 gm. of unsaponifiable material was obtained by extraction of the alkaline hydrolysate with petroleum ether. Sterols were first removed by treatment with 1 gm. of digitonin in 80 per cent ethanol and the digitonides exhaustively washed with petroleum ether. The precipitate weighed 0.735 gm. and contained  $0.02 \pm 0.02$  atom per cent deuterium.

The combined filtrate and washings from the above precipitation were evaporated to dryness *in vacuo* and the solid residue repeatedly extracted with boiling ether. From the ethereal solution 0.169 gm. of non-precipitable unsaponifiable material was recovered. This was transferred to a side arm test-tube equipped with a ground glass joint into which a "cold finger" was mounted, and the contents distilled for 32 hours at 0.02 mm. pressure at 95°. 43 mg. of a pale yellow oily distillate were collected and redistilled in the same apparatus at 80°. 27 mg. were collected in 15 hours. Under these conditions, cetyl alcohol, if present at all, would be expected to be found in the distillate. In order to free the distillate of non-alcoholic contaminants, the alcohol hemisuccinates were prepared by refluxing the volatile material with 0.120 gm. of succinic anhydride in 1 cc. of dry pyridine. The reaction mixture was taken up in ether and washed free of pyridine with dilute  $\text{H}_2\text{SO}_4$ . The hemiesters were extracted from the ether layer with aqueous  $\text{Na}_2\text{CO}_3$ , the water layer removed, acidified, and the hemiesters reextracted with fresh ether. The ether was washed, dried, and evaporated to dryness, to give 22 mg. of a pale yellow oil, semisolid at room temperature. Further purification was deemed impracticable, in view of the small quantity of material. The product was, therefore, analyzed for deuterium and proved to contain  $0.72 \pm 0.03$  atom per cent, calculated on the basis of cetyl alcohol.

#### SUMMARY

1. Cetyl and octadecyl alcohols containing deuterium were prepared from the corresponding fatty acids. The acetates of these alcohols were fed, in small amounts, to normal rats. Both substances were readily absorbed and rapidly converted into saturated fatty acids of the same chain length. In addition,

a portion of the cetyl alcohol was converted into stearic acid and a part of the octadecyl alcohol into palmitic acid.

2. The reverse reaction, the conversion of fatty acids into higher alcohols, has been studied in rats fed deuteropalmitic acid. The fraction of higher aliphatic alcohols obtained from the feces had a deuterium content indicative of its origin from palmitic acid.

3. The results are taken to mean that these alcohols are normal intermediates in fat metabolism, and their possible rôle in the process of elongation of the carbon skeleton has been discussed.

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## PHOSPHORYLATION OF GLUCOSE IN KIDNEY EXTRACT\*

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The reversible enzymatic equilibrium, glycogen +  $\text{H}_3\text{PO}_4 \rightleftharpoons$  glucose-1-phosphate, makes it likely that glucose, in order to be converted to glycogen, must first be phosphorylated. In looking for a system in which phosphorylation of glucose might be studied, our attention was directed to a series of papers by Kalckar (1-3). He found that addition of glucose, inorganic phosphate, and fluoride to a cell-free extract of kidney resulted in the formation of hexosediphosphate, a reaction which occurred under aerobic conditions only and was clearly connected with the oxygen uptake of the extract. Addition of substances (such as glutamic, citric, and succinic acids) which increased the respiration of the extracts also increased the phosphorylation of glucose. This aerobic mechanism of phosphorylation was also demonstrated in a few experiments with liver extract.

In the experiments reported in the present paper an attempt was made to find the components of the phosphorylating system in kidney by reactivating extracts which had lost activity either by aging or by dialysis. In this manner it was found that at least two coenzymes, adenylic acid and cozymase, one specific ion,  $\text{Mg}^{++}$ , and an oxidizable substrate (a dicarboxylic acid) are essential components of the system.

### EXPERIMENTAL

#### *Methods*

Active extracts could be prepared from beef, lamb, pig, cat, or rabbit kidney, obtained within 15 minutes after death of the

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animal. In most cases kidney cortex of fed rabbits was used. About 10 gm. of tissue were ground at 0° with an equal volume of 0.05 to 0.2 M phosphate buffer of pH 7.7 (9 moles of  $\text{Na}_2\text{HPO}_4$  to 1 mole of  $\text{KH}_2\text{PO}_4$ ) in a stainless steel homogenizer of the type described by Potter and Elvehjem (4). The resulting paste was centrifuged for 3 minutes at 2000 R.P.M. and the turbid supernatant extract was used, either immediately or after aging at 5–10° for a period of 4 to 24 hours. In some cases the extract was dialyzed in thin collodion membranes against 4 liters of phosphate buffer (of one-half the concentration used for extraction) for 4 hours at 0° with vigorous outside stirring.

1 cc. portions of the extract were mixed with 0.4 cc. of the additions described in Tables I to X and the mixtures were shaken in Warburg vessels at 37° in an atmosphere of  $\text{O}_2$ . The measurement of  $\text{O}_2$  consumption was started after an equilibration period of 5 minutes. After incubation the samples were fixed with trichloroacetic acid and the filtrates were analyzed for inorganic phosphate by the method of Fiske and Subbarow (5). In some cases the filtrates were also analyzed for phosphoglyceric acid by the method of Rapoport (6), fructosediphosphate as fructose by the method of Seliwanoff as modified by Roe (7),<sup>1</sup> and malic and fumaric acids by the methods of Straub (8). Free glucose was determined by the method of Shaffer and Somogyi (9) in filtrates prepared by the West method (10). All values in Tables I to X are expressed per 1.4 cc. of reaction mixture.

#### *Undialyzed Extracts*

*Product of Glucose Phosphorylation*—Kalekar (2) found that the product of the aerobic phosphorylation of glucose, fructose, or hexosemonophosphate in extracts containing sodium fluoride was mainly fructosediphosphate. This has been confirmed. The first product of phosphorylation is presumably a hexosemonophosphate ester, but its formation has not yet been demonstrated, because (as may be shown by addition of glucose-1- and glucose-6-phosphate) it is either phosphorylated to fructosediphosphate or split by phosphatases, whereas the diphosphate, once formed, is

<sup>1</sup> The color given by fructosediphosphate in the method of Roe is only 44 per cent of that given by an equivalent amount of free fructose; hence the fructose found per 1 mg. of P is  $180/62 \times 0.44 = 1.28$  mg.

not split very rapidly (Table I). When 1.4 mg. of hexose-6-phosphate P was incubated anaerobically with a water extract of kidney containing 0.02 M NaF,  $0.90 - 0.19 = 0.71$  mg. of inorganic P was formed by dephosphorylation of the added monoester. Aerobically the dephosphorylation of the added monoester was masked by a rapid reesterification of the liberated inorganic P. Fructosediphosphate was formed as indicated by the accumulation of an easily hydrolyzable phosphate ester.<sup>2</sup> Analogous results were obtained with a liver extract in the presence of 0.02 M NaF.

TABLE I

*Dephosphorylation of Hexosemonophosphate in Kidney and Liver Extracts*

Rabbit fasted 24 hours. 1 cc. of H<sub>2</sub>O extract + 1.2 mg. of NaF (0.02 M). Total volume 1.4 cc. Incubation time 25 minutes.

Tissue	Additions	Anaerobic		Aerobic	
		Inorganic P	Additional P split in 10 min. in N H <sub>2</sub> SO <sub>4</sub> at 100°	Inorganic P	Additional P split in 10 min. in N H <sub>2</sub> SO <sub>4</sub> at 100°
		mg.	mg.	mg.	mg.
Kidney	Fixed at once	0.14	0.02	0.14	0.02
	None	0.19	0.01	0.02	0.10
	Hexose-6-phosphate (1.4 mg. P)	0.90	0.03	0.03	0.27
Liver	Fixed at once	0.14	0.01	0.14	0.01
	None	0.16	0.01	0.08	0.04
	Hexose-6-phosphate (1.4 mg. P)	0.73	0.02	0.25	0.22

In some cases we have found a considerable proportion of the product of glucose phosphorylation to be phosphoglyceric acid. In fact in one case (Experiment 1 in Table II) in which the kidney was minced and washed once with 3 volumes of cold water before extraction with phosphate buffer, all of the esterified phosphate was accounted for as phosphoglyceric acid. In the presence of M/30 sodium fluoride<sup>3</sup> (a concentration which inhibited respira-

<sup>2</sup> Fructosediphosphate is hydrolyzed 20 per cent by 10 minutes heating in N H<sub>2</sub>SO<sub>4</sub> at 100°, while hexose-6-phosphate is not appreciably hydrolyzed by this procedure.

<sup>3</sup> Fluoride is known to inhibit the transformation of phosphoglyceric to phosphopyruvic acid and thus prevents dephosphorylation.

tion more than 50 per cent) 0.77 mg. of inorganic P disappeared and 0.74 mg. of phosphoglyceric acid P accumulated, corresponding to 2.15 mg. of glucose; the disappearance of glucose actually observed was 1.95 mg. Without fluoride the disappearance of glucose was not accompanied by a decrease in inorganic P, because the rate of dephosphorylation kept pace with the rate of glucose phosphorylation. This experiment also shows that glucose does not disappear anaerobically.

Experiment 2 in Table II shows that glucose is not phosphorylated during oxidation of glyceraldehyde phosphate to phospho-

TABLE II  
*Formation of Phosphoglyceric Acid*

1 cc. of phosphate extract of washed kidney + 10 mg. of glucose.  
Total volume 1.4 cc. Incubation time 65 minutes.

Experi- ment No.	Experimental conditions	Additions	O <sub>2</sub>  c.mm.	Glucose  mg.	Inor- ganic P  mg.	Phos- phogly- ceric acid P  mg.
1	Oxygen	Fixed at once		10.40	1.95	0.05
		None	1410	8.78	1.97	0.09
	Nitrogen	2 mg. NaF	610	8.45	1.18	0.79
		None		10.65	2.19	
2	Nitrogen	2 mg. NaF		10.98	2.25	
		Fixed at once			1.73	0.08
	Nitrogen	2 mg. NaF + fructosediphosphate (0.31 mg. P) + 10 mg. pyruvic acid			1.74	0.64

glyceric acid, the reaction which is known to be linked with phosphorylation of glucose in yeast extract. It may be seen that addition of fructosediphosphate (which acts as a source of glyceraldehyde phosphate) plus pyruvic acid in the presence of NaF led to the formation of 0.56 mg. of phosphoglyceric acid P, but there was no disappearance of inorganic phosphate.

*Effect of Adenylic Acid and Cozymase*—Both adenylic acid (adenine mononucleotide) and cozymase (diphosphopyridine nucleotide) are necessary for the aerobic phosphorylation of glucose in kidney extracts. This can be shown by aging the extracts for 24 hours at 5–10°, which leads to their inactivation.

Such extracts can be reactivated by addition of coenzymes. In Table III is shown an extract which, when fresh, caused complete esterification of the inorganic P originally present; added adenylic acid had no effect on respiration or phosphorylation. After aging for 24 hours, the oxygen consumption dropped from 420 to 175

TABLE III  
*Effect of Adenylic Acid*

1 cc. of phosphate extract of washed kidney + 2 mg. of NaF + 10 mg. of glucose. Total volume 1.4 cc. Incubation time 65 minutes.

Additions	Fresh extract		24 hr.-old extract	
	O <sub>2</sub>	Inorganic P	O <sub>2</sub>	Inorganic P
	c.mm.	mg.	c.mm.	mg.
Fixed at once.....		0.71		0.85
None.....	420	0.02	175	0.97
Adenylic acid (0.001 M).....	400	0.02	395	0.03

TABLE IV  
*Effect of Adenylic Acid and Cozymase*

1 cc. of phosphate extract of washed kidney + 2 mg. of NaF + 10 mg. of glucose. Total volume 1.4 cc. Incubation time 65 minutes.

Additions	4 hr.-old extract		24 hr.-old extract	
	O <sub>2</sub>	Inorganic P	O <sub>2</sub>	Inorganic P
	c.mm.	mg.	c.mm.	mg.
Fixed at once.....		1.55		1.54
None.....	139	1.66	114	1.69
Adenylic acid (0.001 M).....	266	1.58	241	1.59
Cozymase (0.0005 M).....			141	1.68
Adenylic acid + cozymase.....	308	0.83	259	1.13

c.mm. and no phosphate was esterified. By adding 0.001 M adenylic acid, the respiration was restored to the original level and the phosphate was completely esterified.

The experiment shown in Table IV indicates that cozymase is also essential for phosphorylation. No phosphorylation took place in this extract after it had been kept for 4 hours. Although addition of adenylic acid increased the oxygen consumption, it

did not restore phosphorylation, but when both adenylic acid and cozymase were added, considerable phosphorylation took place. In the same extract after aging for 24 hours, adenylic acid alone and cozymase alone were ineffective; phosphorylation resulted only when both coenzymes were added simultaneously. Adenylic acid had much more effect on respiration than did cozymase.

When the extracts were stored at 0° instead of 10°, coenzymes were not so readily destroyed. One extract was active without addition of coenzymes after 48 hours at 0°. It became inactive

TABLE V

*Effect of Adenosinetriphosphate*

1 cc. of H<sub>2</sub>O extract of kidney + 2 mg. of NaF. Total volume 1.4 cc. Incubation time 65 minutes.

Experimental conditions	Additions	Inorganic P	Additional P split in 10 min. in N H <sub>2</sub> SO <sub>4</sub> at 100°
		mg.	mg.
Nitrogen....	Adenosinetriphosphate, fixed at once	0.35	0.40
" .....	" " + 10 mg. glu-	0.39	0.29
	cose		
Oxygen.....	Adenosinetriphosphate	0.31	0.34
" .....	" " + 10 mg. glu-	0.30	0.35
	cose		
	Inorganic P, fixed at once	1.47	
Oxygen.....	" " + glucose	1.08	

after 96 hours at 0° but could still be reactivated by adenylic acid and cozymase.

Kalckar (2) has shown that adenylic acid, when added in large amounts to kidney extract, is rapidly phosphorylated to adenosinetriphosphate under aerobic conditions. Although this seemed to indicate that adenylic acid acted by accepting inorganic P to form adenosinetriphosphate which then donated its mobile phosphate groups to glucose, he could not demonstrate the latter reaction in minced kidney cortex. We also have found that adenosinetriphosphate does not phosphorylate glucose, either aerobically or anaerobically (Table V). The added adenosinetriphosphate contained 0.4 mg. of acid-labile P, of which 0.11 mg.

disappeared during incubation in nitrogen and 0.06 mg. during incubation in oxygen. Addition of glucose did not have any effect on the disappearance of the labile P, showing that a reaction between glucose and adenosinetriphosphate did not take place. That this extract was able to phosphorylate glucose through another mechanism is shown by the fact that, when inorganic phosphate was added, 0.39 mg. of inorganic P was esterified.

### *Dialyzed Extracts*

When the extracts were dialyzed for 4 hours, they had a very low oxygen consumption and did not phosphorylate glucose. Addition of adenylic acid and cozymase did not restore either

TABLE VI  
*Effect of Boiled Kidney Juice*

1 cc. of phosphate extract + 2 mg. of NaF + 10 mg. of glucose. Total volume 1.4 cc. Incubation time 65 minutes.

Other additions	Undialyzed		Dialyzed	
	Inorganic P	O <sub>2</sub>	Inorganic P	O <sub>2</sub>
	mg.	c.mm.	mg.	c.mm.
Fixed at once. ....	1.48		1.37	
Adenylic acid + cozymase. ....	0.14	495	1.46	67
Boiled kidney juice ( $\approx$ 1 gm. kidney) .....			0.76	322

respiration or phosphorylation (Table VI). However, when a boiled juice of rabbit kidney cortex was added to such an extract, the respiration increased very markedly and the ability to phosphorylate glucose was regained. We tried next to determine what additions would replace the boiled juice in the reactivation of dialyzed extracts. The low oxygen consumption suggested that dialysis had removed most of the oxidizable substrate.

*Reactivation by Mg Ions Plus Oxidizable Substrates*—Kalckar (3) has shown that addition of glutamate, citrate, succinate, fumarate, or malate to undialyzed extracts caused an increase in both respiration and phosphorylation. It can be seen in Table VII that when some of these substrates were added to dialyzed extracts, they were not oxidized very rapidly and caused no phos-



phorylation of glucose. However, when Mg ions, which had no effect when added alone, were added together with *l*(+)-glutamate, the rate of oxidation of glutamate was tripled and 1.44 mg. of P were esterified. Similarly, addition of Mg ions increased the rate of oxidation of  $\alpha$ -ketoglutarate  $4\frac{1}{2}$  times and caused esterification of 1.25 mg. of P. With succinate, Mg ions doubled the respiration and caused esterification of 0.81 mg. of P. The product of phosphorylation was in each case a mixture of fructosediphos-

TABLE VII

*Effect of Mg with Various Substrates*

1 cc. of dialyzed phosphate extract + 2 mg. of NaF + 10 mg. of glucose + 0.3 mg. of adenylic acid\* + 4 mg. of the substrates. Total volume 1.4 cc. Incubation time 65 minutes. 2.81 mg. of inorganic P initially present.

Additions	O <sub>2</sub>	Esterified P
	<i>c.mm.</i>	<i>mg.</i>
0.2 mg. Mg <sup>++</sup> .....	52	0.09
<i>l</i> (+)-Glutamic acid .....	178	0.03
"    "    + Mg <sup>++</sup> .....	550	1.44
$\alpha$ -Ketoglutaric acid .....	162	0.07
"    "    + Mg <sup>++</sup> .....	730	1.25
Succinic acid .....	276	0.07
"    "    + Mg <sup>++</sup> .....	529	0.81
<i>l</i> -Malic acid .....	68	0
"    "    + Mg <sup>++</sup> .....	146	0
Pyruvic acid .....	54	0
"    "    + Mg <sup>++</sup> .....	107	0

\* Addition of cozymase was unnecessary because it was still present in sufficient amount after dialysis.

phate and phosphoglyceric acid. Control experiments in which glucose was omitted from the reaction mixture showed no significant esterification of inorganic P. Table VII also shows that malate<sup>4</sup> and pyruvate were not oxidized very rapidly even with Mg<sup>++</sup> added, and caused no phosphorylation of glucose. Other substrates which did not increase respiration and failed to bring about glucose

<sup>4</sup> This negative result with malate (which is due to inactivation of the labile malic dehydrogenase) was not an invariable finding. Two extracts were obtained in which addition of malate caused a considerable increase in respiration and some phosphorylation of glucose.

phosphorylation were the following: *l*(+)-aspartate, *dl*-alanine, *l*-tryptophane, *l*(+)-lactate, fructosediphosphate, and  $\beta$ -hydroxybutyrate.

Citrate has been found to cause glucose phosphorylation in the same way as glutamate, ketoglutarate, and succinate. It is known that citrate and glutamate are converted to ketoglutarate and that the latter yields succinate in the animal body (11). The respiration obtained with these four substrates in dialyzed kidney extract is due to their oxidation and not to a catalysis of glucose oxidation, since omission of glucose from the reaction mixture does not result in a decrease in respiration. Addition of these substrates to NaF-poisoned extracts in catalytic amounts (0.2 mg. per 1 cc. of extract) has very little influence on respiration and causes no phosphorylation of glucose. It is possible, however, to increase the rate of phosphorylation obtained with small amounts of dicarboxylic acids by addition of lactate and alanine, which indicates that the dicarboxylic acids might be concerned with phosphorylation when they act as catalysts for the oxidation of other substrates.

*Coupling of Succinic Acid Oxidation with Phosphorylation of Glucose*—It is particularly interesting that succinic acid oxidation causes phosphorylation of glucose in extracts in which malic acid has no effect, as shown in Table VII. This means that the dehydrogenation of succinic acid to fumaric acid is connected with the transfer of inorganic phosphate to glucose. An illustrative experiment is shown in Table VIII. Of 4 mg. of succinic acid added, 3.7 mg. were accounted for as an equilibrium mixture of malic and fumaric acids. The low respiratory quotient also indicates that there was very little oxidation past the fumaric-malic stage. Moreover, the observed oxygen consumption<sup>5</sup> (405 - 22 = 383 c.mm.) was very close to the theoretical value (380 c.mm.) for the oxidation of 4 mg. of succinic acid to fumaric acid. In the presence of glucose, 0.40 mg. of P was esterified, a reaction which must have been connected with the dehydrogenation of succinic acid.

<sup>5</sup> In this case, total oxygen consumption was measured by equilibrating the extract for 5 minutes at 37° before tilting in the succinate. This preliminary warming in the absence of substrate has been found to cause a distinct loss of phosphorylating activity without affecting the respiration.

*Rôle of Fluoride*—It has been pointed out that the product of glucose phosphorylation in the presence of fluoride is a mixture of fructosediphosphate and phosphoglyceric acid. When fluoride is omitted, phosphoglyceric acid is rapidly dephosphorylated, as shown in Table II, but an accumulation of fructosediphosphate still takes place. The experiment in Table IX shows the accumu-

TABLE VIII

*Effect of Oxidation of Succinic Acid on Glucose Phosphorylation*

1 cc. of dialyzed phosphate extract + 2 mg. of NaF + 0.3 mg. of adenylic acid. Total volume 1.4 cc. Incubation time 65 minutes. 1.48 mg. of inorganic P initially present.

Additions	O <sub>2</sub>	R.Q.	Esterified P	Fumaric acid	Malic acid
	c.mm.		mg.	mg.	mg.
None.....	22		0.00	0.04	
4 mg. succinic acid + 0.2 mg. Mg <sup>++</sup> ...	405	0.3	0.03	0.68	3.07
4 " " + 0.2 " "					
+ 10 mg. glucose.....	396	0.2	0.40	0.66	

TABLE IX

*Phosphate Ester Accumulation without Fluoride*

1 cc. of dialyzed phosphate extract + 10 mg. of glucose + 0.3 mg. of adenylic acid. Total volume 1.4 cc. Incubation time 65 minutes. 1.35 mg. of inorganic P initially present.

Additions	O	QO <sub>2</sub>	Esterified P	Fructose
	c.mm.		mg.	mg.
None.....	30	1	0	0.02
4 mg. α-ketoglutaric acid + 0.2 mg. Mg <sup>++</sup> .....	1404	41	0.88	0.84
4 " " + 0.2 " "				
+ NaF (M/30).....	656	19	1.31	1.31

lation of 0.88 mg. of esterified P in the absence of fluoride; the fructose reaction indicates that the product is mainly fructosediphosphate. In a similar experiment with an undialyzed extract, of 3.29 mg. of inorganic P added 1.23 mg. of esterified P accumulated in the absence and 2.09 mg. in the presence of fluoride. Kalckar states that addition of fluoride is necessary to prevent the splitting of the phosphorylated products by phosphatases,

but it is evident from the anaerobic experiment in Table I that fluoride does not prevent phosphatase action in kidney and liver extracts. It seems that the chief action of fluoride in this system is the prevention of the breakdown of phosphoglyceric acid by way of phosphopyruvic acid.

When fluoride is omitted, the oxidizing ability of the dialyzed extracts is remarkably high and may surpass that of kidney slices. The dialyzed extract shown in Table IX, which had a spontaneous  $Q_{O_2}$  of only 1 c.mm. per mg. of dry weight of extract per hour, gave a  $Q_{O_2}$  of 41 on addition of ketoglutaric acid plus  $Mg^{++}$ .

*Nature of  $Mg^{++}$  Effect*—Although Mg ions have been known to accelerate a number of enzymatic processes involving transfer

TABLE X  
*Comparison of  $Mg^{++}$  with  $Mn^{++}$*

1 cc. of dialyzed sodium phosphate extract + 10 mg. of glucose + 0.3 mg. of adenylic acid + 2 mg. of NaF. Total volume 1.4 cc. Incubation time 65 minutes. Mg and Mn added as sulfates. 1.34 mg. of inorganic P initially present.

Additions	O <sub>2</sub>	Esterified P
	c.mm.	mg.
None.....	38	0
4 mg. l(+)-glutamic acid.....	220	0
4 " " " +0.2 mg. $Mg^{++}$ .....	448	1.32
4 " " " +0.02 " $Mn^{++}$ .....	520	0.37

of phosphate groups, it was not recognized until recently that Mg ions also have a very strong activating effect on certain dehydrogenases. Von Euler and collaborators (12) have shown that isocitric dehydrogenase is activated by  $Mg^{++}$ , and Ochoa (13) has recently found that pyruvate oxidation in brain is markedly stimulated by  $Mg^{++}$ . In the present experiments the activating effect of  $Mg^{++}$  on isocitric dehydrogenase has been confirmed and a marked activating effect on glutamic, ketoglutaric, and succinic dehydrogenases has been shown.

The small amount of  $Mg^{++}$  still present after 4 hours dialysis is sufficient to permit considerable dehydrogenase activity in some cases, but is not sufficient for phosphorylation of glucose. For example, in Table VII, succinic acid is oxidized at an appreciable

rate without added  $Mg^{++}$ , while phosphorylation occurs only after addition of  $Mg^{++}$ . The experiment in Table X indicates that the effect of  $Mg^{++}$  on glucose phosphorylation cannot be attributed solely to an acceleration of oxidation, since  $Mn^{++}$  has an even stronger effect than  $Mg^{++}$  upon respiration but is much less effective in causing phosphorylation of glucose. It

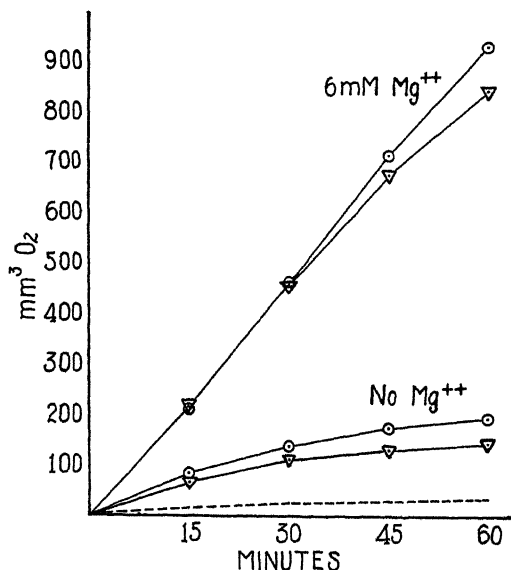


FIG. 1. Effect of  $Mg^{++}$  on oxidation of  $\alpha$ -ketoglutarate. 1 cc. of dialyzed kidney extract + 0.3 mg. of adenylic acid + 10 mg. of glucose + 2 mg. of NaF + 4 mg. of ketoglutaric acid. Total volume 1.4 cc. In curves marked with triangles the reaction mixtures contained 3 mM  $K^+$  and 200 mM  $Na^+$ ; in curves marked with circles 140 mM  $K^+$  and 35 mM  $Na^+$ . The dashed curve shows the respiration without ketoglutaric acid.

should also be pointed out that the activating effect of  $Mg^{++}$  in this system is not due to an overcoming of an inhibitory effect of  $Na^+$ , as Ohlmeyer and Ochoa (14) found for the reaction between phosphopyruvic acid and adenylic acid. This is shown in Fig. 1, in which the  $Mg^{++}$  effect is just as great in the presence of an excess of  $K^+$  as in an excess of  $Na^+$ . One may assume that  $Mg^{++}$  has two effects as a component of the phosphorylating system of

kidney; it accelerates the oxidation of the dicarboxylic acids and it is necessary for the reaction by which phosphate is transferred to glucose.

#### DISCUSSION

It is of interest to compare the aerobic mechanism for glucose phosphorylation in kidney with the anaerobic mechanism in other systems. Phosphorylation of glucose in yeast is dependent on simultaneous dehydrogenation of glyceraldehyde phosphate by cozymase and takes place anaerobically if a hydrogen acceptor (acetaldehyde) is available. The same "coupled reaction" has been shown to take place in a number of tissues (erythrocytes (15), tumor (16), retina (17), brain (18), embryo (19)) which break down glucose anaerobically. In all these cases the reaction described by Negelein and Brömel (20) occurs; glyceraldehyde phosphate reacts with inorganic phosphate and is converted to glyceric acid-1,3-diphosphate, one phosphate group of which is transferred to glucose over the adenylic acid system. Although kidney extracts are able to form phosphoglyceric acid anaerobically by dismutation of glyceraldehyde phosphate and pyruvic acid, a simultaneous phosphorylation of glucose cannot be demonstrated.

It is evident that phosphorylation of glucose in kidney extracts is not dependent on the dehydrogenation of glyceraldehyde phosphate but on the oxidation by molecular oxygen of any one of a number of dicarboxylic acids. It is possible that it is the oxidation of succinic to fumaric acid (a reaction common to the oxidation of all of the effective substrates) which brings about phosphorylation of glucose. In Szent-Györgyi's theory of cellular respiration the transport of hydrogen involves the pair succinic  $\rightleftharpoons$  fumaric acid as a link to the cytochrome system. The oxidation of a number of substrates involving fumaric acid catalysis might therefore cause phosphorylation.

There is good evidence that the aerobic mechanism here described is also present in liver. This mechanism serves for the phosphorylation of glucose and is thus connected with glycogen synthesis. It also serves for the phosphorylation of fructose and glycerol, and explains why in previous experiments with liver slices (21, 22) a conversion of these substances to glucose occurred only under aerobic conditions.

## SUMMARY

1. Kalekar's finding that the esterification of glucose with inorganic phosphate in cell-free kidney extract occurs only under aerobic conditions has been confirmed. The product which accumulates in the presence of sodium fluoride is a mixture of fructosediphosphate and phosphoglyceric acid. Anaerobically no phosphorylation occurs either during dismutation of added triose-phosphate and pyruvic acid or upon addition of large amounts of adenosinetriphosphate.

2. The following are essential components of the aerobic phosphorylating system, as determined by reactivation of aged or dialyzed extracts: (a) an oxidizable substrate, such as citrate, glutamate, ketoglutarate, or succinate; (b) at least two coenzymes, namely adenylic acid and cozymase; adenylic acid has a marked stimulating effect on both respiration and phosphorylation; (c) Mg ions, which are necessary for both the oxidation of the substrates mentioned and for the transfer of phosphate to glucose.

3. Malate, pyruvate, lactate, fructosediphosphate, *dl*-alanine, *l*-tryptophane, *l*(+)-aspartate, and  $\beta$ -hydroxybutyrate are not rapidly oxidized in dialyzed extracts and do not cause phosphorylation of glucose.

4. The activating effect of succinate in extracts which are not reactivated by malate indicates that the transfer of inorganic phosphate to glucose is connected with the dehydrogenation of succinic to fumaric acid. It is suggested that a number of substrates whose oxidation involves fumaric acid catalysis may be concerned with glucose phosphorylation.

We are indebted to Dr. Meyerhof and Dr. Ohlmeyer for a sample of pure cozymase and to Dr. Lipton for the  $\alpha$ -ketoglutaric acid.

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## CHOLINESTERASES IN THE BLOOD OF MAN

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A significant discrepancy in the results of investigators who have studied the effects of various conditions on the rate of splitting of choline esters by enzyme preparations has apparently been generally overlooked. Studies of whole blood or tissue extracts by Plattner and Hintner (1), Galehr and Plattner (2), and others, at low substrate concentrations and with bioassay methods, gave activities greater (by about 10 times) than indicated by extrapolation of the data for horse (3-5) and human (6) serum preparations obtained at high substrate concentrations and with chemical assay methods (7, 8). The fact that the reaction kinetics for all types of enzyme preparations and experimental conditions were compatible with the Michaelis-Menten relationship (9-11) has overshadowed the differences between the enzyme preparations. Stedman *et al.* (12, 13) measured cell enzyme activity because they realized that the serum enzyme could not account for the activity of whole blood, particularly at low substrate concentrations, but did not make clear that the cell enzyme activity observed was insufficient to account for the reported results at low substrate concentrations unless the cell enzyme behaves differently with changed experimental conditions than does serum enzyme. Stedman *et al.* (12-15) compared the activities of serum and cell enzyme, using the method for serum enzyme, but did not establish that the activity of whole blood is equal to the sum of their separate effects. We have found this to be true, if care is taken to keep the conditions of titration the same for both types of preparations and to express the results on a comparable basis in terms of the amount of blood from which each type of preparation is derived.

In addition to establishing that the enzymes from serum and

from blood cells are qualitatively different, our experiments showed that the greater part of the acetylcholine, which may be injected into the blood stream or may diffuse into it from tissues innervated by cholinergic fibers, is hydrolyzed by the enzyme within the blood cells. Galehr and Plattner (2) came to this conclusion from direct measurements of the relative activity of blood cells and serum by bioassay at small substrate concentrations, but their findings have been neglected because they were in apparent contradiction to the more precise measurements made at high substrate concentrations.

In the present work, the serum and cell enzymes of human blood were found to be unlike in their behavior in four types of experiments: (1) a study of the activities with acetylcholine and four of its methyl derivatives as substrates, (2) a study of the effect of altering pH, (3) measurements of the activities at different concentrations of acetylcholine, (4) determination of the activating effect of sodium chloride on hydrolysis.

#### *Apparatus and Methods*

Estimations of enzymatic activity by biological assay of residual acetylcholine ion in reaction mixtures (*e.g.*, see Englehart and Loewi (16), Plattner and Hintner (1), and Ginsberg, Kohn, and Necheles (17)), have inherently low precision and are much more time-consuming and expensive than those depending on chemical methods. The eight chemical methods (6-8, 13, 18-21) of enzyme assay which have been used depend on the fact that one of the products of the reaction is acetic acid. They differ in details of temperature, pH, and substrate kind and concentration, but have in common the fact that relatively high substrate concentration is necessary to provide sufficient acid on hydrolysis for chemical detection.

Continuous titration, with a glass electrode-vacuum tube potentiometer sensitive to 0.01 pH unit (Beckman pH meter), was selected as the chemical technique of the greatest precision, simplicity, and versatility, with least danger of interference from added reagents or manipulation. Since Glick (6) had not described his technique of electrometric titration with the glass electrode at the time this work was started, the conditions selected for titration differ in some respects from his.

After study had shown that no material increase in accuracy could be attained under other conditions, the pH (7.40) and temperature (37°) for routine titrations were chosen to approximate physiologic values. The titration volume was 25 ml. and the standard substrate concentration was 0.160 M. The titration vessel was a jacketed glass tube warmed by circulating water, and the contained solution was kept well stirred with a glass paddle. Electrodes were immersed in the solution, their design and small size insuring their rapidly coming to temperature equilibrium, and the calomel half-cell liquid junction was then rinsed afresh to maintain the saturation of the KCl solution.

After the warmed reagents were mixed, the pH was quickly adjusted and maintained within  $\pm 0.10$  pH unit by small additions of 0.02 N NaOH from a 5 ml. burette. Readings to the nearest second of time required for consumption of measured amounts of alkali furnished data from which total hydrolysis rates could be calculated. Readings were continued until the desired accuracy was obtained. Usually 10 to 20 minutes gave values duplicable within  $\pm 2$  per cent. "Rates" were calculated as volumes of 0.0200 N NaOH used in 20 minutes, in conformity with the Stedman (7) procedure. Blanks, calculated from the pH-alkaline hydrolysis relationships given in the experimental part, were subtracted from total hydrolysis rates. The value of the blank rate under standard conditions, with acetylcholine ion as substrate, was 0.085 ml.

The iodides of acetylcholine and its derivatives were used for preparing the substrate solutions, as they are stable and non-hygroscopic. In contradiction to the results of Roepke (4), it was found by trial that iodide ion does not interfere. The 0.0200 M stock of acetylcholine iodide ordinarily used was adjusted to pH 4.0. This solution changes only just detectably at room temperature during a month, presumably by oxidation, since calculation by extrapolation from reaction rate constants measured at higher pH values indicated the hydrolysis due to  $\text{OH}^-$  ion to be less than 1 per cent per year at this pH and a temperature of 37°.

### *Preliminary Experiments*

1. *Enzyme Stability*—Many investigators have found that blood enzyme preparations keep well, but it appeared desirable

to verify this conclusion for our particular conditions, and to extend the time interval. Accordingly, venous blood samples, laked in water containing 1:100,000 phenylmercuric acetate and kept at 7°, were tested at intervals under standard conditions. The results are given in Table I and it may be seen that they are quite stable. The sample of December 18, 1936, Enzyme G, may have decreased in potency, but these differences do not provide definite proof of deterioration. Samples of the same serum (human)

TABLE I  
*Enzyme Stability*

Enzyme	Dilution	Test date	Rate	Deviation from mean
		1936		per cent
E	1:25	Sept. 10	0.81	1.2
		" 11	0.80	0.0
		" 23	0.79	-1.2
F	1:5	Nov. 6	0.434	-1.85
		" 7	0.447	1.08
		" 14	0.432	-2.31
		" 14	0.448	1.31
		" 16	0.444	0.41
		" 27	0.448	1.31
G	1:5	Dec. 18	0.560	4.09
		" 19	0.553	2.79
		" 23	0.533	-0.93
		" 31	0.548	1.86
		1937		
		Jan. 5	0.522	-2.98
		" 12	0.511	-5.02

kept under aseptic conditions, undiluted and diluted 1:5 with water containing 1:100,000 phenylmercuric acetate, gave titration values of 0.448 and 0.443, respectively, after 21 days at 7°.

2. *Inactivation by Heat*—Controls were run with heated enzyme preparations from the blood of two persons to determine whether other constituents of the preparations would contribute to the blank values. Serum, or washed red blood corpuscles laked in water, heated to 60° for 10 minutes, when titrated at pH 7.4, 8.0, 8.6, and 9.2 under otherwise standard conditions, gave rates identical to those of control runs without enzyme under the same

conditions, within the experimental error. However, whole blood diluted 1:5 with saline containing the usual preservative, or washed corpuscles suspended in the saline to 5 times the volume of the blood from which they were taken, and heated to the same extent, were not completely inactivated, about 14 and 26 per cent of the original activity of these preparations remaining at pH 7.4. On longer heating (60°, 50 minutes) they became completely inactive.

*3. Relative Activity of Serum, Blood Cells, and Whole Blood under Standard Conditions*—The contribution of serum and of

TABLE II  
*Distribution of Enzyme in Blood*

	Subject	From 0.2 ml. blood		Serum + cells	Whole blood, 0.2 ml.	Ratio, serum to blood
		Serum	Cells			
Males	G. A.	0.50	0.30	0.80	0.80	0.62
	H. M.	0.55	0.31	0.86	0.90	0.61
	C. B.	0.47	0.35	0.82	0.84	0.56
	G. P.	0.49	0.36	0.85	0.87	0.56
Females	M. K.	0.43	0.34	0.77	0.74	0.58
	V. K.	0.42	0.35	0.77	0.83	0.52
	M. B.	0.39	0.28	0.67	0.65	0.60
	G. W.	0.53			0.89	0.67
Average . . . . .				0.79	0.80*	0.590

\* The figure for G. W. was left out of this average.

cells to the enzymatic activity of whole blood was measured for eight adults, four male and four female. The data are shown in Table II, where the "rates" given are calculated to show the activity per 0.2 ml. of defibrinated whole blood. The figure for serum plus cells is the sum of the separate titration values.

To prepare the blood fractions tested, venous blood was added to 4 parts of saline, shaken with glass beads, and allowed to stand several hours to precipitate fibrin, and then strained through gauze into calibrated centrifuge tubes. The sample for the whole blood titration was removed, the remainder centrifuged well, and then the cell and total volumes measured. The diluted serum was separated; the cells were washed twice with saline and then sus-

pended in water sufficient to make the volume equal to the measured total volume. Aliquots of these separated materials were taken for the titrations. The error of estimation of volume in the tubes used is about  $\pm 3$  per cent, so the results should be accurate within  $\pm 5$  per cent. The enzyme in the serum is a moderately constant proportion of the total (one normal person, studied later, formed an exception) and washing the cells did not cause loss of appreciable quantities of enzyme. Our data agree with those of Stedman and Stedman (13) on the magnitude and extent of variation of the ratios of serum to whole blood activities, if the values for their Subject 1 are omitted, but we are somewhat uncertain how to interpret their data, since their values for serum plus corpuscles do not equal the values for whole blood. Our figures for the sum of serum and cell activities equal those for whole blood, although it will be seen later that this is true only because of the fortunate selection of conditions of titration in which the difference in NaCl content of the enzyme preparations had only a slight effect on their determined activities.

4. *Surface Hydrolysis on Blood Cells*—Cellular debris from a laked washed blood cell enzyme preparation was removed by centrifuging and washed with saline, then tested for enzymatic activity. There was no detectable increase in hydrolysis over the control amount, and the activity of the precipitate was certainly less than 2 per cent of that of the laked cell preparation from which it was derived. Without doubt, the enzyme of the blood cells is a soluble substance (or substances) which does not diffuse through the intact cell wall. Galehr and Plattner (2, 22) suggested that the hydrolysis of acetylcholine ion by red blood cells might be a surface catalysis analogous to that which they observed when using charcoal. Our experiment, together with the fact that the blood cell enzyme is completely destroyed by moderate heating, makes it quite certain that any non-specific hydrolysis by these preparations is negligibly small.

#### *Experiments with Varied Substrates and Effect of Changes of pH*

*Alkaline Hydrolysis*—Both the hydroxyl ion and enzymatic hydrolyses of acetylcholine, its  $\alpha$ - and  $\beta$ -methyl derivatives, and the erythro and threo forms of its  $\alpha$ ,  $\beta$ -dimethyl derivatives were measured at various pH levels. The iodides of all the acetyl-

choline derivatives used were prepared from the corresponding dimethylamino alcohols by treatment with acetic anhydride and then methyl iodide (Table III).

Table IV shows the results of experiments on alkaline hydrolysis rate, at various pH values, with 20 ml. of 0.02 N substrate solution in a total volume of 25 ml. at 37.0°, in terms of the specific reaction rate constant  $k$ , calculated from the first order equation (25),  $-dc/dt = kc$ , or  $\ln c_0/c = kt$ , and  $k = \text{rate}/10^3 \times m$ , wherein rate is the titration figure in ml. of 0.02 N alkali for 20 minutes,  $m$  the average of beginning and end millimolal amounts of substrate (the changes of the substrate concentrations were small, so

TABLE III  
*Preparation of Iodides of Acetylcholine Derivatives*

Compound of acetylcholine iodide	M.p.	Dimethylamino alcohol prepared from
	°C.	
$\alpha$ -Methyl .....	137-138	2-Dimethylamino-1-propanol, b.p. 140-141°*
$\beta$ -Methyl .....	125-126	1-Dimethylamino-2-propanol, b.p. 124-125°
Erythro- $\alpha,\beta$ -dimethyl ...	150	3-Dimethylamino-2-butanol, b.p. 152-153°†
Threo- $\alpha,\beta$ -dimethyl .....	108	3-Dimethylamino-2-butanol, b.p. 141-142°†

\* Karrer *et al.* (23).

† Wilson and Lucas (24).

that the error introduced by taking the arithmetic average instead of the exact value, namely the difference between the initial and final concentrations divided by the natural logarithm of their ratio, was negligible),  $c_0$  is the initial concentration, and  $c$  the substrate concentration at  $t$  minutes after the beginning of observation. The equations given were obtained from the data by least squares. With acetylcholine itself, observations were also made with concentrations 0.5 and 2 times that used for the data given in Table IV, and the specific reaction rate constants were the same, well within the limits of error of the titration.

Three batches of acetylcholine iodide (Hoffmann-La Roche) were used; one had been freshly recrystallized from ethanol. All



TABLE IV

*Alkaline Hydrolysis*

Temperature 37°; total volume 25 ml.; compound, mm.

Compound	Amount	pH	"Rate"	$k \cdot 10^3$	$\text{Log}_{10} k$	Equation, $\text{log}_{10} k =$
Acetylcholine	0.399	7.40	0.0922	0.230	-3.64	1.006 (pH) - 11.111
	0.398	7.40	0.0860	0.216	-3.67	
	0.400	7.40	0.0892	0.224	-3.65	
	0.346	7.70	0.142	0.411	-3.39	
	0.395	8.00	0.329	0.833	-3.08	
	0.395	8.20	0.579	1.47	-2.83	
	0.392	8.20	0.513	1.31	-2.88	
	0.386	8.60	1.389	3.60	-2.44	
	0.367	9.00	3.25	8.86	-2.05	
	0.380	9.00	3.44	9.06	-2.04	
$\alpha$ -Methyl acetylcholine	0.376	9.00	3.18	8.46	-2.07	0.9776(pH) - 10.870
	0.398	7.40	0.0966	0.243	-3.615	
	0.349	7.40	0.0737	0.211	-3.676	
	0.397	7.40	0.0933	0.235	-3.629	
	0.375	7.40	0.0829	0.221	-3.655	
	0.394	8.00	0.351	0.891	-3.050	
	0.351	8.00	0.300	0.855	-3.068	
	0.392	8.00	0.373	0.951	-3.022	
	0.196	8.00	0.1945	0.993	-3.003	
	0.388	8.60	1.305	3.36	-2.473	
$\beta$ -Methyl ace- tylcholine	0.357	8.60	1.171	3.28	-2.484	0.9535(pH) - 11.208
	0.383	8.60	1.380	3.61	-2.443	
	0.372	9.20	4.83	13.0	-1.886	
	0.399	7.40	0.030	0.075	-4.124	
	0.397	8.00	0.101	0.255	-3.594	
	0.377	8.00	0.095	0.252	-3.599	
	0.393	8.60	0.364	0.927	-3.033	
	0.385	9.20	1.500	3.90	-2.409	
	0.400	7.20	0.066	0.165	-3.778	0.6045(pH) - 8.035
	0.398	7.40	0.116	0.292	-3.535	
Erythro- $\alpha, \beta$ - dimethyl acetylcholine	0.400	7.90	0.245	0.612	-3.212	
	0.398	7.90	0.261	0.649	-3.182	
	0.396	8.00	0.275	0.695	-3.158	
	0.397	8.50	0.598	1.51	-2.822	
	0.386	8.60	0.440	1.14	-2.943	
	0.392	8.70	0.536	1.37	-2.863	
	0.391	8.90	0.822	2.10	-2.678	
	0.373	9.20	1.158	3.10	-2.508	
	0.381	9.40	1.98	5.20	-2.284	

TABLE IV—*Concluded*

Compound	Amount	pH	"Rate"	$k \cdot 10^3$	$\text{Log}_{10} k$	Equation, $\text{log}_{10} k =$
Threo- $\alpha$ , $\beta$ - dimethyl acetylcholine	0.400	7.10	0.020	0.049	-4.308	$0.8499(\text{pH}) - 10.356$
	0.398	8.00	0.131	0.329	-3.483	
	0.398	8.00	0.114	0.286	-3.543	
	0.380	8.00	0.086	0.226	-3.645	
	0.396	8.50	0.296	0.748	-3.126	
	0.394	8.60	0.286	0.726	-3.139	
	0.394	9.10	0.940	2.38	-2.624	
	0.390	9.20	1.11	2.84	-2.546	
	0.386	9.20	1.244	3.22	-2.492	

the data obtained with these three solutions are given in Table IV. A solution which had been stored 6 months at room temperature in the dark in a rubber-stoppered Pyrex flask was also titrated, and gave a value of  $k$  at pH 7.4 which was 4.1 per cent below that calculated from the equation given. This is within the limit of error for a determination at this pH.

These data furnish evidence that the relationship between compound concentration, hydroxyl ion concentration, and hydrolysis rate is, in fact,  $-dc/dt = k' \cdot c \cdot (\text{OH}^-)$ , or that the reaction follows the expected bimolecular law (25), for acetylcholine, for the  $\alpha$ -methyl-substituted compound, and perhaps for the  $\beta$ -methyl compound also.<sup>1</sup>

In preliminary experiments we observed that when fresh solutions of the  $\alpha$ -methyl compound were used the rate constant  $k$  gradually decreased from the value at the start of the run to a value which was 22 per cent lower in an hour at 37° and pH 7.4; while with solutions which were aged several days at room temperature, or overnight at 37°, the rate constant started at the lower value and remained constant for more than an hour. To obtain more uniform results, all solutions of these four compounds were thereafter incubated 18 hours at 37° in tightly stoppered Pyrex flasks before use. The acid liberated during this incubation in no

<sup>1</sup> Our interest in data for the alkaline hydrolysis of these compounds was primarily to obtain reliable figures to correct the enzymatic hydrolysis titrations for this factor, and we did not investigate the anomalous behavior (departure of the slopes of the equations of Table IV from 1) of the dimethyl-substituted compounds.

case amounted to more than that which would have been released by the hydrolysis of 2 per cent of the compound present. The incubated solution of  $\alpha$ -methyl acetylcholine iodide, stored at

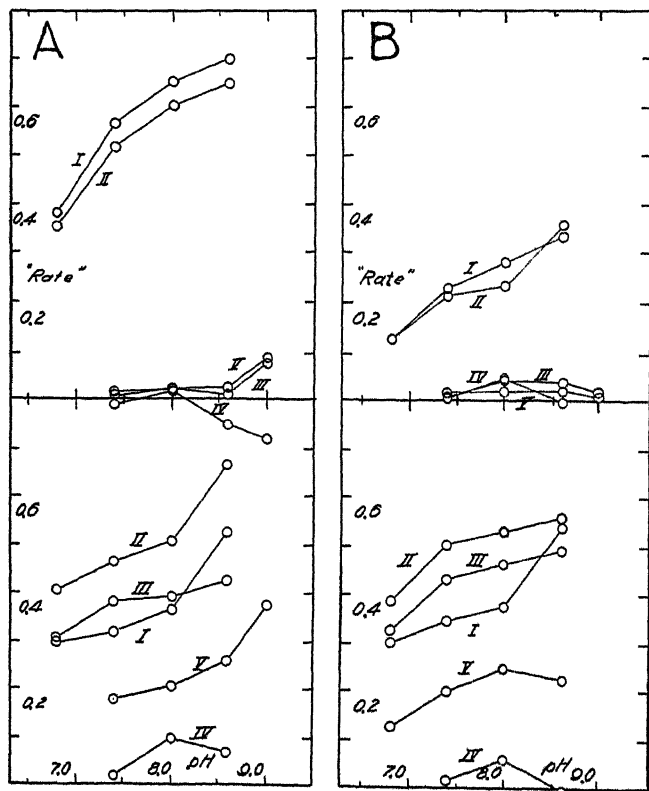


FIG. 1. Point diagrams (A and B) showing enzymatic hydrolysis *versus* pH for separated serum and blood cell enzymes from bleedings of two individuals. The substrates for the respective curves were I, acetylcholine iodide; II,  $\alpha$ -methyl acetylcholine iodide; III,  $\beta$ -methyl acetylcholine iodide; IV, erythro- $\alpha,\beta$ -dimethyl acetylcholine iodide; V, threo- $\alpha,\beta$ -dimethyl acetylcholine iodide. The upper part of each diagram shows serum values and the lower, cells.

room temperature in the dark, gave the same value of  $k$  at pH 8.0, within 1 per cent, 6 days later. All experiments on the enzymatic hydrolysis of the various compounds were completed within 13 days of making up the solutions.

*Enzymatic Hydrolysis*—For experiments on the enzymatic hydrolysis of the various substrates, the enzyme preparations were made up by the technique described under "Preliminary experiments," Section 3, except that no whole blood sample was taken.

The enzymatic rates corrected for pipette error, and in the case of serum reduced to the amount from 0.2 ml. of defibrinated whole blood, are shown in Fig. 1. It should be noted that the accuracy of the titrations decreases rapidly above pH 8.0, because the correction for alkaline hydrolysis becomes such a large fraction of the total hydrolysis, and also because the unavoidable slight fluctuations of pH around the value selected for titration have a much more marked effect on alkaline than on enzymatic hydrolysis.

Whole blood titrations had been run with this same set of substrates on the same two persons a year previous to our discovery of the difference between serum and cell enzymes. The sums of the activities of serum and of cell enzymes were in excellent agreement with the former titrations. Formerly it was considered necessary to assume that the enzymes from these persons were qualitatively different, in order to account for the observed differences in behavior with the different substrates. The data for the fractions show, however, that the difference between them lay wholly in the different proportion of serum and cell enzyme. The person who provided the enzyme of *B* (Fig. 1) is the one referred to under "Preliminary experiments," Section 3, whose serum to whole blood ratio is abnormally low.

These data show a striking qualitative difference between the enzymes of serum and of blood cells. Serum enzyme has almost no activity toward the  $\beta$ -methyl-substituted compounds under these titration conditions, whereas the cell enzyme is more active toward  $\beta$ -methyl acetylcholine than toward acetylcholine.

#### *Experiments at Various Substrate and Sodium Chloride Concentrations*

Enzyme preparations for these experiments were made from venous blood by the technique described. After preliminary experiments showed a marked difference between the sum of serum and blood cell activities and that of whole blood in the region of 0.01 mM of acetylcholine ion per 25 ml., 1 ml. of 0.85 per cent NaCl

was added to some cell enzyme titrations to bring the salt concentration approximately up to that of the serum. By thus maintaining equivalent conditions in all titrations, the greatest discrepancy (about 12 per cent), at any point in the concentration range, between the activity of whole blood and the sum of serum and cell activities was within the limit of experimental error. The greatest effect of this small concentration of NaCl (0.034 per cent) amounted to an increase of rate of 64 per cent over that without the addition. At the standard test level of acetylcholine ion concentration it was only 16 per cent. In some of the experiments, NaCl sufficient to give a final concentration of 0.85 per cent (4.0 ml. of 5.1 per cent, or 5.3 per cent, as required) was added to the mixtures. All titrations were carried out in a 25 ml. volume at pH 7.40, and at 37°.

Experiments confirmed the observation of Stedman and Stedman (13) that laking the cells does not alter their activity, and extended it to apply over the range of acetylcholine ion concentrations studied, from 0.016 M to approximately  $4 \times 10^{-6}$  M (the greatest deviation in any experiment was 7 per cent). To avoid confusion, only the data for the runs in which the cells were laked are shown.

The enzymatic rates, corrected for pipette error, and in the case of serum reduced to the amount from 0.2 ml. of defibrinated whole blood, are shown in Fig. 2. The hydrolysis rates at levels below 0.004 millimole are not corrected for choline ion inhibition. They were measured in mixtures containing 0.004 millimole of acetylcholine iodide initially. The inhibition by choline ion is negligible at the higher concentration levels, on account of the high ratio of acetylcholine ion to choline ion at the time of the start of each run when readings were taken. The actual initial rates, obtained by back extrapolation in a few cases, were not appreciably different from the figures given. A dotted line in Fig. 2 marks the 0.004 millimole per 25 ml. level (0.00016 M). Very similar results were obtained when these experiments were repeated with blood from another person.

The activity of serum and of cells toward acetylcholine ion is affected differently by alteration of its concentration, the cell enzyme being several times more active at low substrate concentrations, and being inhibited by excess substrate. The results

are in accord with the experiments of other investigators who have studied the behavior of tissue extracts, of serum, and of whole blood at various substrate concentrations, if one assumes that the tissue preparations used by Plattner and Hintner (1) contained a considerable proportion of enzyme having a characteristic like that of the human blood cell enzyme. Also, 0.85

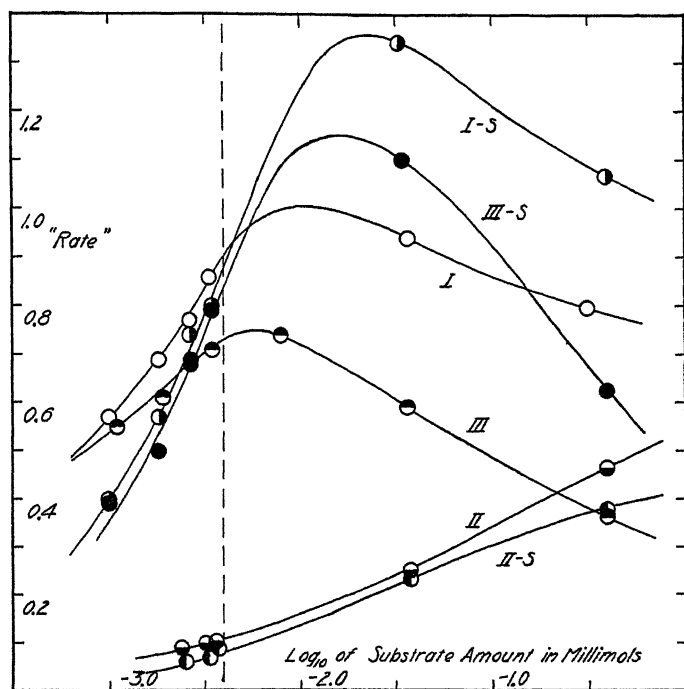


FIG. 2. Enzyme from subject C. B., October 9, 1937. Curve I, whole blood; Curve II, serum; Curve III, laked blood cells; all at approximately 0.034 per cent NaCl. The curves marked -S are for the corresponding enzyme preparations tested in the presence of 0.85 per cent NaCl.

per cent NaCl affects the cell enzyme markedly (average maximum change for three enzyme preparations tested, 82 per cent) over part of the concentration range, and the serum enzyme much less and in the opposite direction (greatest change observed, 17 per cent).

For the sake of completeness, the same enzyme preparations

were examined for the effect of pH variation, with and without NaCl additions. The enzymatic rates, corrected for pipette error, and in the case of serum reduced to the amount from 0.2 ml. of defibrinated whole blood, are shown in Fig. 3. These titrations were run under standard conditions, except as indicated.

Because of the possibility that inhibition by liberated choline ion (4, 5) may greatly affect the shape of the curves obtained at low substrate concentrations (below 0.0040 millimole per 25 ml.), and because the accuracy of our method is not great, due to the

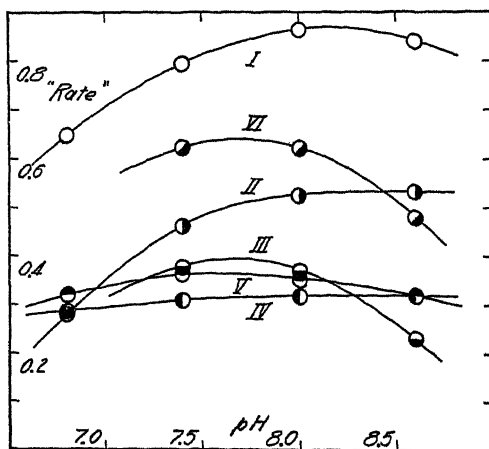


FIG. 3. Enzyme from subject C. B., October 9, 1937. Curves I, II, and V represent whole blood, serum, and cells respectively, at 0.034 per cent NaCl; Curves III and VI, serum and cells at 0.85 per cent NaCl; Curve IV, cells with no added NaCl.

buffer action of the enzyme preparations, under these same conditions, we have not attempted to calculate the enzyme-substrate equilibrium constant for the blood cell enzyme from our data. Some experiments done with a whole blood preparation indicated that choline ion inhibition of cell enzyme is appreciable, but we have not considered it worth while to present the data because of the difficulties of interpretation introduced by using the mixed enzymes.

#### DISCUSSION

Since Ginsberg, Kohn, and Necheles (17) have shown that washed pus cells are inactive toward acetylcholine ion, it is prob-

able that the activity in blood cell enzyme preparations derives from the erythrocytes. We are unable to suggest any special physiological function that these cells possess which requires the presence of cholinesterase, other than that also attributed to the serum cholinesterase of preventing the circulating blood from transmitting a local activity of cholinergic nerve endings to remote sites. Since by far the greater part of the blood activity under normal physiologic conditions appears to reside in the cells, it is possible that the plasma serves chiefly as a reservoir for the distribution and equilibration of tissue cell enzyme. In this connection it is interesting that Glick (26) found the cholinesterase of the superior cervical ganglion of the cat behaved like serum enzyme in its substrate-concentration-activity relationship.

Our findings suggest that caution must be used in the interpretation of cholinesterase estimations found in the literature with reference to physiologic function or to alterations in various physiologic or disease conditions.

#### SUMMARY

1. A simple, constant acidity, continuous titration technique, with a glass electrode, furnishes an accurate method of determining the cholinesterase activity of blood samples over a considerable range of experimental conditions.

2. A striking qualitative difference between the enzymes of serum and of cells of human blood is shown in their relative activities upon acetylcholine and its methyl derivatives.

3. With regard to alteration of acetylcholine substrate concentration, the enzymes of serum and of cells of human blood behave quite differently; at low acetylcholine concentrations the cell enzyme is of chief importance for the hydrolysis of acetylcholine by whole blood.

4. The serum and cell enzymes of human blood vary differently in their activities with regard to changes of pH and salt content of their environment.

5. Since the serum and cell enzymes of whole human blood differ importantly, studies on either enzyme alone are inadequate to value the rôle played by the cholinesterase activity of whole blood.



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## CONVENIENT PROCEDURES FOR THE PREPARATION OF ANTIHEMORRHAGIC COMPOUNDS

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The following procedures for the synthesis of a series of related compounds of established antihemorrhagic activity, including vitamin K<sub>1</sub>, were developed as a result of experimentation undertaken with the object of simplifying and standardizing known processes reported from the author's laboratory and elsewhere. The methods given are believed to be more convenient and more rapid than any previously described. All of the procedures except the last one listed were submitted for trial to a group of students half-way through a course in elementary organic chemistry with entirely successful results.

*2-Methyl-1,4-Naphthoquinone*—The reaction time can be decreased from 3 days (1) or 36 hours (2) to a little over 1 hour. Steam distillation (1) of the product is unnecessary.

A solution prepared by dissolving 50 gm. of chromic anhydride in 35 cc. of water and diluting with 35 cc. of glacial acetic acid is added in portions from a separatory funnel to a solution of 14.2 gm. of  $\beta$ -methylnaphthalene in 150 cc. of glacial acetic acid contained in a 600 cc. beaker; the solution is stirred with a thermometer during the addition. The temperature is allowed to rise to 60° and then checked at that point by ice cooling and maintained at 60° throughout the addition of the reagent, which can be completed in about 10 minutes. When the temperature begins to drop spontaneously, the solution is heated on the steam bath (85–90°) for 1 hour to complete the oxidation and diluted with about 500 cc. of water. After the material is stirred for a few minutes, the precipitated yellow quinone is collected on a Büchner funnel and washed thoroughly with water. The crude material while still moist is crystallized from 40 cc. of methanol

(without filtering), giving 6.5 to 7.3 gm. (38 to 42 per cent) of 2-methyl-1,4-naphthoquinone, m.p. 105–106°, suitable for the following preparations. (If the quinone is required for biological work, it should be recrystallized from methanol, and a trace of insoluble material removed by filtration.) The procedure has given good results for the oxidation of as much as 100 gm. of the hydrocarbon.

*2-Methyl-1,4-Naphthohydroquinone*—The following method of reduction is more rapid than either of the two procedures previously described (2) and is applicable to both small and large scale operations.

The quinone (2 gm.) is dissolved in 35 cc. of ether by warming and the solution is poured into a separatory funnel and shaken with a fresh solution of 4 gm. of sodium hydrosulfite in 30 cc. of water.<sup>1</sup> After the mixture is shaken for a few minutes, the solution passes through a brown phase (quinhydrone) and becomes pale yellow. After removal of the aqueous layer the ethereal solution is shaken with a mixture of 25 cc. of saturated sodium chloride solution and 1 to 2 cc. of saturated hydrosulfite solution to remove the bulk of the water and filtered by gravity through a paper moistened with ether and about one-third filled with anhydrous magnesium (or sodium) sulfate. The filtrate is evaporated until nearly all of the ether has been removed, cooled, and treated with petroleum ether (b.p. 20–40°). The hydroquinone separating as a white or grayish powder is washed with petroleum ether and air-dried; yield, 1.9 gm. (94 per cent).

*Phthiocol*—The following procedure embodies various improvements in the synthesis discovered by Madinaveitia (3) and recently elaborated by Anderson and Creighton (4). This synthesis is better adapted to preparative purposes than certain others reported (1, 5, 6) and in its present form may well be superior to the process briefly indicated by Kuroda (7). The present improvements, which reduce the reaction times from several hours to a few minutes with increase in the yield, consist in preparing

<sup>1</sup> With large quantities it is not necessary to use enough ether to dissolve the quinone or to employ such a large excess of reducing agent. Satisfactory amounts per gm. of quinone are 3 cc. of ether and a solution of 0.7 gm. of sodium hydrosulfite in 2 cc. of water. In this case a longer period of shaking is required.

methylnaphthoquinone oxide with hydrogen peroxide (2) in place of hypochlorite solution (3, 4) and in isomerizing it with concentrated rather than dilute sulfuric acid.

2-Methyl-1,4-naphthoquinone (1 gm.) is dissolved in 10 cc. of alcohol by warming and the solution allowed to stand while the second reagent is prepared by dissolving 0.2 gm. of anhydrous sodium carbonate in 5 cc. of water and adding (cold) 1 cc. of 30 per cent hydrogen peroxide solution. The quinone solution is cooled under the tap until crystallization begins, the peroxide solution is added all at once, and the mixture is cooled. The yellow color of the quinone is at once discharged, giving a colorless or pale yellowish solution. On addition of about 100 cc. of water and cooling in ice, *2-methyl-1,4-naphthoquinone oxide* separates as colorless crystals, m.p. 93.5–94.5° (pure, 95.5–96.5°); yield, 0.97 gm. (89 per cent).

The dry oxide (1 gm.) is treated with 5 cc. of concentrated sulfuric acid (without cooling) and the mixture stirred if necessary to produce a homogeneous deep red solution. After 10 minutes this is cooled in ice and slowly diluted with 20 cc. of water. The precipitated phthiocol may be collected and washed (0.93 gm., m.p. 171–172°) and crystallized by dissolving it in methanol (25 cc.) containing 5 drops of concentrated hydrochloric acid (4), clarifying the solution with Norit, concentrating, and diluting to the saturation point. Alternately, the yellow suspension is washed into a separatory funnel and the product extracted with a mixture of 25 cc. each of benzene and ether. The organic layer is dried over magnesium sulfate and evaporated to a volume of about 10 cc. for crystallization. The total yield of pure phthiocol, m.p. 172–173°, is 0.84 to 0.88 gm. (84 to 88 per cent). Purification by steam distillation or by extraction from ether-benzene with sodium carbonate solution is unnecessary.

*Vitamin K<sub>1</sub> (2-Methyl-3-Phytyl-1,4-Naphthoquinone)*—Considerable further experimentation on the synthetic method previously described (8) has led to the development of the following simplified procedure in which the reaction time is reduced from 36 to 4 hours. Observations to be reported elsewhere with Dr. Max Tishler indicate that the chief by-product is a ketonic substance formed by an independent reaction and not a naphthotocopherol and hence that the reason advanced (8) for employ-

ing a very large excess of methylnaphthohydroquinone is no longer tenable. A moderate excess seems desirable for best utilization of the phytol, but on a small scale this can be controlled to a point where the recovery operation can be dispensed with.

A mixture of 1.48 gm. of phytol, 1.5 to 2.0 gm. of 2-methyl-1,4-naphthohydroquinone, 1 gm. of anhydrous oxalic acid,<sup>2</sup> and 10 cc. of commercial dioxane is warmed and stirred until the solids are dissolved, the flask is stoppered with a cork carrying a thermometer, and the solution heated for 4 hours<sup>3</sup> on the steam bath at 90–95°. The somewhat orange solution is cooled to 30°, washed into a separatory funnel with 40 cc. of ether, and washed with two 40 cc. portions of water to remove dioxane and oxalic acid. To remove unchanged hydroquinone the solution is shaken vigorously with a freshly prepared solution of 2 gm. of sodium hydrosulfite in 40 cc. of 2 per cent aqueous potassium hydroxide and 10 cc. of saturated sodium chloride solution (which helps break the resulting emulsion). After the pressure is released through the stop-cock, the funnel is kept stoppered until the layers separate as a precaution against oxidation. The yellow liquor is drawn off and the extraction repeated a second and third time, or until the alkaline layer remains practically colorless. The faintly colored ethereal solution is then dried over magnesium sulfate, filtered into a tared flask, and evaporated on the steam bath, eventually while being evacuated at the water pump. The total oil, which becomes waxy on cooling, amounts to 1.7 to 1.9 gm.

When the brown mass is treated with 10 cc. of petroleum ether (b.p. 20–40°) and the mixture is boiled and stirred, the material soon disintegrates, giving a suspension of white solid. This is washed into paired centrifuge tubes with 10 to 20 cc. of fresh petroleum ether and centrifugation is carried out after cooling thoroughly in ice. The brown supernatant liquor is decanted into the original tared flask, the white sludge of vitamin K<sub>1</sub> hydroquinone is stirred with fresh solvent to an even suspension, and the mixture is cooled, centrifuged, and separated by decantation as before. Evaporation of the liquor and washings gives

<sup>2</sup> Prepared by heating the dihydrate at 130–140° until dry. This condensing agent can be replaced by 1 cc. of 85 per cent phosphoric acid.

<sup>3</sup> The yield is slightly lower when the heating is continued for only 3 hours and is not improved by extending the period to 5 or 6 hours.

1.1 to 1.3 gm. of residual oil, the difference affording a check on the yield of vitamin. The washed white sludge is dissolved in 10 to 15 cc. of absolute ether and a little Norit is added if the solution is at all pink or dark. Silver oxide (1 gm.) and magnesium sulfate (1 gm.) are added and the mixture shaken for 20 minutes to effect oxidation. The yellow solution is then filtered into a tared flask and evaporated on the steam bath, traces of solvent being removed at the water pump. The residue is a light yellow oil consisting of pure vitamin K<sub>1</sub>; yield, 0.55 to 0.65 gm. (24 to 29 per cent). Undue exposure to light should be avoided when the material is in the quinone form.

*Sodium 2-Methyl-1,4-Naphthohydroquinone Disulfate*—The method of preparing this salt in a pure condition briefly indicated in a previous paper (9) has been considerably improved and standardized as follows:

While the mixture is being cooled in ice, 0.5 cc. of chlorosulfonic acid is added by drops from a burette to a solution of 1 cc. of pyridine in 5 cc. of carbon tetrachloride. To the resulting salt suspension is added a cold solution prepared by dissolving 0.5 gm. of 2-methyl-1,4-naphthohydroquinone in 1 cc. of pyridine by gentle warming, diluted with 5 cc. of carbon tetrachloride. The mixture is worked with a stirring rod and then heated for 15 minutes on the steam bath with occasional stirring. When cooled in ice, the oil can be caused to stiffen and adhere to the walls, when the liquor is removed by decantation. Fresh wash solvent is added and the mixture heated to boiling and stirred; after ice cooling the solvent is again poured off. The pale yellow, viscous oil consisting of the pyridine salt of the reaction product and pyridine hydrochloride is stirred with 3 cc. of water to give a turbid solution, and after the rod is washed with 0.5 cc. of water 0.65 gm. of sodium bicarbonate is added, a few drops of ether being used at the end to break the foam. The solution is poured into a small dry separatory funnel, rinsed with 0.5 cc. of water, and is then extracted with ether, the solvent being removed by suction through a pipette. The pale yellow solution is treated with 2 cc. of pyridine and then, while being cooled as required to keep the temperature at about 25°, 2 cc. of 50 per cent sodium hydroxide solution (50 gm. of pellets in 100 cc. of water) are run in by drops with good mixing. The funnel is stoppered and shaken, after which the mixture separates into an upper brown

layer and a heavier, pale yellow layer containing inorganic salts. The latter is drawn off and discarded, and the upper layer is shaken with ether, cleaning the stem of the funnel while the latter is inverted. The ether layer containing extracted pyridine is removed by pipette. The pale brown layer is run into a dry flask and treated with 20 cc. of absolute ethanol, which is run in through the funnel. The reaction product separates slowly as a white powder, and after thorough cooling in ice this is collected, washed with absolute ethanol and with ether, and air-dried.

The crude salt is treated with 5 cc. of cold methanol, when the bulk of the product dissolves to a turbid solution containing a little suspended inorganic salts. A little Norit is added and after warming to effect coagulation the solution is filtered by suction, 2 cc. of methanol being used to wash the filter, and the filtrate is diluted with 35 cc. of absolute ether to precipitate the product as a white powder. The collected product after air drying is essentially pure and satisfactory for use; yield, 0.5 to 0.7 gm. (42 to 49 per cent). A sample further purified by repeated precipitation from methanol lost 1.6 per cent of its weight on being dried to constant weight in a vacuum at room temperature. Analysis of the dried salt (by Lyon Southworth) showed it to be the dihydrate.

*Analysis*—Calculated for  $C_{11}H_8O_8S_2Na_2 \cdot H_2O$ ,  $H_2O$  8.70; found (dried at  $100^\circ$  in a vacuum),  $H_2O$  8.53. The anhydrous salt on exposure to moist air reverted to the dihydrate, for the gain in weight corresponded to a water content of 8.98 per cent.

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# THE EFFECT OF DENATURING AGENTS ON MYOSIN\*

## I. SULFHYDRYL GROUPS AS ESTIMATED BY PORPHYRINDIN TITRATION

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Myosin is a protein distinguished by its very high viscosity and intense double refraction of flow. It is also one of the few proteins which possess titratable sulfhydryl groups even in the native state; in the presence of certain denaturing agents the number of such groups is very greatly increased. Study of the action of a large number of denaturing agents on myosin has revealed certain phenomena quite different from those accompanying denaturation of the "globular" proteins. The present paper deals with the effect of various agents on sulfhydryl groups, as determined by the porphyrindin titration; Paper II deals with the effects of the same agents on viscosity, double refraction of flow, and solubility. These studies have revealed that myosin is profoundly altered by many very mild reagents not ordinarily classed as denaturing agents for proteins. The effects of these reagents are particularly clearly revealed by measurements of viscosity and double refraction; but their effects on sulfhydryl groups are also significant, and have been reported in this paper.

### EXPERIMENTAL

#### *Preparation of Myosin*

*Rabbit Myosin*—This was prepared from leg muscles of rabbits by a process somewhat modified from the earlier procedure of Edsall (5). The animals were killed by exsanguination under

\* A preliminary account of part of the work reported in this paper and Paper II has already been given (6).



anesthesia. The blood from the hind legs was drained out and the muscles were rapidly chopped up and ground as fine as possible. The ground muscle was then immediately transferred into 10 to 15 parts of a solution containing potassium chloride (0.5 *N*) and sodium bicarbonate (0.03 *N*) at 4°. The mixture was gently stirred mechanically for 2 hours in the cold, strained through cheese-cloth, and filtered through paper pulp on a Buchner funnel.

The filtrate, which generally showed strong double refraction of flow in a layer 2 cm. in depth when tested by means of the apparatus described in Paper II, was now brought to a pH near 6.3 by the slow addition of dilute acetic acid, with constant stirring. At this pH the protein solution was appreciably more opalescent than before the addition of acid, but no precipitate formed.<sup>1</sup> It was now diluted with 5 to 10 volumes of cold distilled water; the flocculent precipitate which formed was allowed to settle out overnight in a cold room, and the supernatant fluid was siphoned off. The precipitate was centrifuged in the cold, washed once or twice by centrifuging with cold distilled water, and dissolved by the addition of finely ground potassium chloride crystals with constant stirring. Usually the final concentration of KCl needed for complete solution at this pH was 0.4 to 0.5 *N*. Dilution with 2 volumes of cold distilled water readily reprecipitated the protein. The precipitate was again centrifuged, washed with cold distilled water, and redissolved in KCl. The solutions so obtained generally showed strong double refraction of flow.<sup>2</sup>

The myosin solutions, dissolved in KCl at a pH of approximately

<sup>1</sup> On the acid side of pH 6.0 myosin is insoluble in salt solutions, and may rapidly become irreversibly denatured, at least in part. On the other hand, the precipitate formed on dilution with water does not settle well unless the pH is below 6.5 (unpublished observations of H. O. Singher in this laboratory). At pH near 6.3 the conditions are nearly optimal for the precipitation of undenatured myosin.

<sup>2</sup> Occasionally preparations were found in which double refraction had largely or completely disappeared. The reasons for this alteration were not discovered, but the observations recorded in Paper II indicate some of the many possible mechanisms which may have been involved. We wish to emphasize the importance of testing the protein solution for double refraction at every stage of the preparation, as this measurement apparently affords the most sensitive criterion of the undenatured state of the protein.

6.2, were treated with a few drops of toluene and held at 4°. If preserved free from bacterial contamination, they still showed intense double refraction after several weeks or even months, but the experiments described in this paper were in general carried out as soon as possible after the protein was prepared.

*Protein concentration* was determined by the micro-Kjeldahl procedure of Koch and McMeekin (8), the nitrogen content of the solution after digestion being determined by nesslerization. The protein content was calculated from the nitrogen content, Bailey's (3) figure of 16.6 for the percentage of nitrogen in myosin being used. Bailey's value was obtained on myosin preparations which had been precipitated, and then thoroughly washed with alcohol and ether before being dried. Under these conditions, the dried myosin is obtained as a fine white powder. On the other hand, some preparations made in this laboratory were prepared by washing myosin (precipitated from salt solution by dilution) with water, and then removing the water by evaporation *in vacuo* at low temperature over phosphorus pentoxide. Under these circumstances, the dry myosin is yellowish, glassy in appearance, and difficult to grind. The nitrogen content was found to be only 15.7 per cent. Probably dry myosin so prepared contains some lipids which are removed by Bailey's alcohol-ether treatment. The difference in nitrogen content between the two types of preparation is in harmony with this explanation, as is also the statement of Weber (14) that myosin contains approximately 10 per cent of lipid material. Todrick and Walker (13) have reported an even lower nitrogen content of 15.3 per cent for myosin. For the present, we consider Bailey's figure of 16.6 per cent as the most satisfactory basis for calculation of protein content. Sharp (12) has recently reported the figure of 16.8 per cent, in close agreement with Bailey.

#### *Estimation of Sulfhydryl Groups in Proteins*

These groups were estimated by titrating the protein with porphyrindin (9, 7). A fuller and more explicit description of this method than that given in the earlier papers appears desirable here.

The method rests upon two assumptions; namely, (1) that a positive nitroprusside reaction in a protein solution is given only by —SH groups, and (2) that porphyrindin is reduced in a neutral

protein solution, at room temperature, within 1 to 2 minutes, only by —SH groups. It is known that the carbonyl group will give a positive nitroprusside reaction, but the permanency and tint of the color so developed are in marked contrast to the rapidly fading color given by simple mercaptans and by proteins. It is known that tyrosine and phenols in general in alkaline solution will affect porphyrindin, but the rate of this reaction is much slower than the very rapid reduction by mercaptan groups. Moreover, the color developed by the dye in the presence of phenols is an orange-yellow, in contrast to the practically colorless condition of the reduced form of the dye brought about by simple mercaptans and by proteins which presumably contain —SH groups. The possibility cannot be excluded that there may exist in protein molecules other groups of an unknown nature which give a positive nitroprusside reaction and reduce porphyrindin to the colorless leuco form. However, in the absence of further evidence, we have chosen tentatively to regard as sulfhydryl the reactive groups in proteins which respond to these tests.

The reason for using both criteria is the general observation that proteins which do not give a positive nitroprusside reaction do not reduce porphyrindin, whereas those proteins which give the former reaction also reduce the dye. The parallel feature of the two types of reaction is further emphasized by the readily observable fact that as one adds porphyrindin progressively to a solution of an appropriate protein, the initial nitroprusside reaction becomes weaker and finally negative. At this point the dye has been completely reduced and the solution is colorless. Excess of dye beyond this point results in persistence of the blue color for a period of several minutes to half an hour, depending upon the protein. On the basis of these observations the conditions for the estimation of the sulfhydryl groups in proteins have been chosen. Briefly stated, the amount of added dye just necessary to cause a negative nitroprusside reaction in the protein was taken to be a measure of the sulfhydryl groups present.

The method is quite simple and rapid. In each of several test-tubes of 10 ml. capacity are placed 2.0 cc. of the protein solution. To the first is added a certain volume of the porphyrindin solution, and the tube is shaken for not more than 1 to 2 minutes. A drop of dilute ammonia is added, followed by about 0.5 ml. of dilute

sodium nitroprusside. If the reaction is positive, the second tube is treated with a larger quantity of porphyrindin. A positive nitroprusside reaction in this case is followed by the addition to the third tube of a still larger quantity of porphyrindin. The procedure is thus carried on by successive approximations until just the amount of dye is added which will bring about a loss in nitroprusside reaction. The end-point by this method is easily reproducible. Both before and after the titration of the protein the dye solution is standardized against a known solution of cysteine. The amount of dye consumed by the protein in order to bring it to the specified end-point is expressed in terms of its equivalent of cysteine for 100 gm. of protein.

The protein sulfhydryl groups estimated as above may properly be designated as "titratable." A few proteins in the *native* state contain such titratable groups.<sup>3</sup> Among them is myosin. When

<sup>3</sup> Balls and Lineweaver (4) have recently questioned the general applicability of the nitroprusside and porphyrindin reactions to the identification of sulfhydryl groups in proteins. Their objection to this method was based on the failure of crystalline native papain to give a positive nitroprusside reaction or to reduce porphyrindin, although the protein apparently reacted with iodoacetic acid. It is clear from these authors' description that native papain behaves toward nitroprusside and porphyrindin exactly as do native egg albumin, edestin, excelsin, and globin (7). These proteins, as well as papain, do not react with the above reagents in the *native* state, although the proteins do contain —SH groups which are revealed by denaturation. The recent experiments of Anson (1) illustrate this point further, for he states that ferricyanide, although it reacts with denatured egg albumin, does not react with native egg albumin. Iodine and iodoacetamide, on the other hand, do so. Anson's value for —SH groups in egg albumin, as determined by titration with dilute ferricyanide in the presence of Duponol PC, agrees well within the limits of experimental error with Greenstein's value (7) obtained by titration with porphyrindin in the presence of concentrated guanidine hydrochloride. (Their values are 1.21 and 1.24 per cent respectively, expressed as percentage of cysteine.) If a similar agreement is obtained for other proteins, it will go far to establish the validity of both methods for estimation of the maximal number of titratable sulfhydryl groups in proteins. The apparent conflict between the various points of view may be readily resolved by drawing a distinction between two types of sulfhydryl groups in proteins. Groups of the first type do not react with nitroprusside, porphyrindin, or ferricyanide, but do react with iodine and iodoacetic acid. Groups of the second type react with all the reagents mentioned. Many native proteins such as egg albumin, edestin, and papain apparently possess sulfhydryl groups of the first

urea, guanidine hydrochloride, or methylguanidine hydrochloride was added to a solution of myosin, an appreciably greater number of titratable sulfhydryl groups was revealed.

### *Results*

Todrick and Walker (13) estimated the sulfhydryl groups in native myosin by determining the amount of phenol indo-2, 6-dichlorophenol dye reduced over a period of 12 hours. The result of these investigators, calculated as per cent cysteine, is 0.27. Mirsky (10) estimated the sulfhydryl groups in myosin by comparing the cysteine content of the protein hydrolysate before and after treatment of the protein with iodoacetate. His value for native rabbit myosin was 0.31 per cent cysteine. After treatment with trichloroacetic acid, the cysteine apparently rose to about 0.57 per cent.

Our first experiments were concerned with estimating the amount of sulfhydryl groups in native myosin dissolved in KCl and in myosin dissolved in urea and in guanidine hydrochloride (Table I). The values given for the protein in solutions of urea and guanidine hydrochloride include the cysteine found in the native protein. The proportion of cysteine is apparently independent of the protein concentration. The value in the native protein, 0.42 per cent cysteine, is somewhat higher than that given by Mirsky (10) and is appreciably higher than that given by Todrick and Walker (13).<sup>4</sup> In solutions of urea and of guanidine hydro-

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type which on denaturation of the protein are transformed into groups of the second type. It is difficult to say at present whether this transformation is effected by a change in the spatial configuration of the protein (2) or whether denaturation causes a breaking of certain loose bonds involving sulfhydryl. In this connection the work of Schubert (11) on the compounds of aldehydes and ketones with mercaptans is suggestive. Schubert finds that the loosely bound hemimercaptals fail to give a nitroprusside reaction but do reduce iodine. It would be premature to postulate labile hemimercaptal linkages in the native protein molecule, but the parallelism is striking. The work of Balls and Lineweaver (4) indicates that the sulfhydryl group which is apparently necessary for specific activity of papain is of the first type.

<sup>4</sup> The —SH content of myosin solutions slowly diminishes on standing. In one preparation the cysteine equivalent of the —SH groups had fallen from 0.41 per cent to 0.30 per cent in 2 weeks at 4°.

TABLE I

*Sulfhydryl Groups in Native Rabbit Myosin and in Myosin Treated with Urea and Guanidine Hydrochloride*

2.0 cc. of protein solution were used in each determination. The dye was made up in  $1.16 \times 10^{-3}$  M concentration (0.0325 per cent) in 0.5 M KCl solution. Urea and guanidine hydrochloride were added to the protein solution so that the concentration in each case was 16.7 mM per gm. of  $H_2O$ .

Preparation No.	Protein concentration  <i>per cent</i>	Per cent cysteine found in		
		KCl, 0.5 M	Urea	Guanidine HCl
II	0.60	0.41	0.62	1.19
I	0.85	0.40	0.64	1.12
II	1.00	0.41	0.66	1.18
III	0.63	0.43	0.65	1.12
III	0.93	0.42	0.63	1.11
IV	2.30	0.39		1.12
IV	1.90	0.46	0.68	1.18
V	0.78	0.40	0.64	1.16

TABLE II

*Effect of Varying Concentrations of Guanidine Hydrochloride on Myosin*

2.0 cc. of a 0.85 per cent solution of protein were used in each determination. The dye concentration was  $1.16 \times 10^{-3}$  M (0.0325 per cent) in 0.5 M KCl.

Guanidine HCl added  <i>mM per gm. <math>H_2O</math></i>	Dye  <i>mM <math>\times 10^4</math></i>	Cysteine  <i>per cent</i>
0.0	2.96	0.40
0.5	2.96	0.40
1.0	4.22	0.57
2.1	6.07	0.82
4.2	7.48	1.01
6.3	8.06	1.09
8.4	8.12	1.12
10.4	8.12	1.12
12.5	8.12	1.12
14.6	8.12	1.12
16.7	8.12	1.12

chloride, the amount of sulfhydryl groups increases markedly, the increase being greater in guanidine hydrochloride.

Myosin was further investigated in solutions of varying concen-

tration of guanidine hydrochloride. The strong effect of this reagent is revealed in Table II, the results being comparable to those obtained with other proteins.

The data obtained on the different preparations of rabbit myosin were extremely consistent. In five different preparations the value found in potassium chloride solution was  $0.42 \pm 0.03$  per cent cysteine and in concentrated guanidine hydrochloride (above 8 M) it was  $1.15 \pm 0.04$  per cent cysteine. In one preparation (No. IV) double refraction and viscosity were unusually low and the solution could be concentrated to an extent quite unusual in myosin preparations. Nevertheless, the sulfhydryl content both in potassium chloride solution and in guanidine hydrochloride was found to be the same as that in the other preparations.

The effects of several derivatives of urea and guanidine hydrochloride and the effects of several inorganic salts on myosin are reported in Table III. Methylguanidine hydrochloride is as effective as the parent substance, whereas the methylated ureas produce a distinctly different effect.

Ammonium chloride, methylammonium chloride, dimethylammonium chloride, arginine monohydrochloride, O-methylisourea hydrochloride, hydrazine monohydrochloride, and acetamide reduce the content of titratable sulfhydryl groups to 0.<sup>5</sup> This effect was also produced by N-methylurea in high concentration and by glycine in a concentration as low as 0.01 M. None of these substances produced any such effect on cysteine or other simple —SH compounds.<sup>6</sup> The effect has been found repeatedly and invariably in all the myosin preparations tested. Addition of concentrated guanidine hydrochloride to the mixture of myosin with glycine or ammonium chloride or arginine monohydrochloride immediately restored the full content of titratable sulfhydryl groups found in the completely denatured protein (see Table I).

In connection with the studies reported in Table III, the experiment was tried of adding to the native protein solution just

<sup>5</sup> The protein solution under these conditions gives a negative nitroprusside reaction and fails to reduce porphyrindin.

<sup>6</sup> Ammonium salts are usually employed to enhance the color developed by the nitroprusside reagent with —SH groups. It is obvious that the use of these salts with various tissues calls for a certain amount of caution, particularly if it is desired to estimate the sulfhydryl content of such tissues.

sufficient porphyrindin to oxidize the free —SH groups completely. When urea, guanidine hydrochloride, or methylguanidine hydrochloride was added to this oxidized protein, the amount of free —SH groups which appeared exactly corresponded to the differences found between the native and denatured protein in any

TABLE III  
*Effect on Myosin of Various Substances*

2.0 cc. of a 0.78 per cent solution of protein were used in each determination. The dye was used in  $1.16 \times 10^{-3}$  M (0.0325 per cent) concentration in 0.5 M KCl.

Reagent	Concentration of reagent	Cysteine
	<i>mM per gm. H<sub>2</sub>O</i>	<i>per cent</i>
0.5 M KCl in H <sub>2</sub> O.....		0.40
Urea.....	16.7	0.64
N-Methylurea.....	10 —16.7	0
	1.7	0.40
O-Methylisourea HCl.....	16.7	0
Guanidine HCl.....	16.7	1.16
“ H <sub>2</sub> SO <sub>4</sub> .....	1.1	0.40
Methylguanidine HCl.....	16.7	1.16
Arginine monohydrochloride.....	0.5	0
Glycine.....	0.01– 1.0	0
Ammonium chloride.....	1.7	0
Methylamine HCl.....	1.7	0
Dimethylamine HCl.....	1.7	0
Hydrazine monohydrochloride.....	1.7	0
Acetamide.....	16.7	0
KI.....	1.0	0.40
KSCN.....	1.0	0.40
MgCl <sub>2</sub> .....	1.0	0.40
CaCl <sub>2</sub> .....	1.0	0.40
LiCl.....	1.0	0.40

one of these solvents. Addition of N-methylurea and O-methylisourea hydrochloride to the solution of oxidized native myosin produced no increase in —SH groups. Thus, if one titrates the free —SH groups of the native protein, then adds the denaturing reagent, and resumes the titration, the total amount of dye consumed is the same as if the denaturing agent had been added at the beginning.



A number of salts—KI, LiCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, KSCN—were tested for their effect on the sulfhydryl groups with negative results, the —SH content in all these solutions being the same as in potassium chloride.

#### DISCUSSION

The best analytical data on the sulfur of the muscle proteins are those of Bailey (3) who gives the total sulfur of rabbit myosin as 1.10 per cent and the methionine sulfur as 0.73 per cent.

In concentrated guanidine hydrochloride, the porphyrindin titration yields the equivalent of 1.14 per cent of cysteine, or 0.305 per cent cysteine sulfur in myosin. This is approximately five-sixths of the difference between total sulfur and methionine sulfur in myosin; 95 per cent of the total sulfur can thus be accounted for as cysteine and methionine sulfur.<sup>7</sup>

Myosin apparently contains a fairly high proportion of free and titratable sulfhydryl groups in the native state (Table I). The amount of these groups on the one hand is greatly increased by treatment of the protein with guanidine hydrochloride and, on the other hand, is reduced to 0 by treatment with certain ammonium derivatives. Is the protein "denatured" by either procedure or by both? Obviously, a definition of denaturation based on either procedure would be arbitrary.

We have no explanation to offer at present as to what becomes of the —SH groups of native myosin when the latter is treated with ammonium salts. They reappear, together with additional —SH groups, when the protein is treated with guanidine hydrochloride. Thus a mixture of myosin with glycine, like native egg albumin, shows no titratable —SH groups, but does so after treatment with guanidine. Myosin is remarkable in that the appearance or the disappearance of sulfhydryl groups may be caused by the use of reagents in themselves non-reactive toward mercaptans. The amount of reagent required to effect disappearance is very

<sup>7</sup> It may be pointed out that the value of the porphyrindin titration, expressed as cysteine sulfur, for all the proteins which have been investigated (7) has never been greater than the value for the total sulfur minus methionine sulfur. If the reagent reacted with other groups in addition to sulfhydryl groups, one would not expect such a relation to be generally found.

much less than that required to effect appearance of sulfhydryl groups.

The total —SH content of myosin is the sum of the sulfhydryl groups titrated when the protein is in the native state plus the additional sulfhydryl groups revealed when the protein is denatured in concentrated guanidine hydrochloride solution. The total amount of dye consumed is apparently the same whether one titrates the native protein first, and then adds the guanidine salt and resumes titration, or whether the titration is conducted from the start in the presence of guanidine salt. In the former case it is clear that porphyrindin has oxidized the free sulfhydryl groups characteristic of the native myosin to a form which is not affected by the subsequent addition of guanidine salt. That this oxidized form may be disulfide seems probable, but is not proved. Porphyrindin readily and quantitatively oxidizes cysteine to cystine—whether it oxidizes sulfhydryl in the protein to disulfide remains for further investigation.

#### SUMMARY

1. An improved method for the preparation of myosin is described.

2. The free sulfhydryl groups of rabbit myosin have been estimated by the use of porphyrindin and the results are expressed in terms of cysteine for 100 gm. of protein. A critical discussion of the significance of the porphyrindin titration is given.

3. The cysteine equivalent of the —SH groups in native rabbit myosin is 0.42 per cent. In concentrated urea this figure rises to 0.65 per cent, and in concentrated guanidine hydrochloride or methylguanidine hydrochloride to 1.15 per cent. Treatment of the native protein with various ammonium derivatives and amino acids reduces titratable —SH to 0. Addition, however, of guanidine hydrochloride to these latter solutions brings out all the sulfhydryl groups characteristic of the fully denatured protein.

4. If one titrates the free —SH groups of the native protein, and then adds guanidine hydrochloride and resumes the titration, the total amount of dye consumed is the same as if the denaturing agent had been added at the beginning.

5. 95 per cent of the total sulfur of myosin may be accounted for as methionine plus cysteine sulfur.

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# THE EFFECT OF DENATURING AGENTS ON MYOSIN

## II. VISCOSITY AND DOUBLE REFRACTION OF FLOW

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Myosin is notable for its high viscosity and intense double refraction of flow. These properties depend upon the extreme asymmetry of the molecule, and are intimately related to its function as the chief structural component of the muscle fiber. Alterations in these properties afford a very sensitive indication of change in the state of the myosin molecule, and such changes are very readily brought about, not only by the commonly recognized denaturing agents, but by a great variety of organic molecules, and even by many simple inorganic electrolytes. Many of these substances act on myosin with great speed and at low concentrations. Invariably their effect is to diminish double refraction of flow until it is undetectable by the method thus far employed by us, and to lower the viscosity of the myosin solution, while also reducing the variation of apparent viscosity with the rate of shear. Whether these phenomena indicate denaturation of myosin is a question of terminology. In this communication we shall describe the phenomena, indicate how far they resemble, and how far they differ from, the phenomena associated with denaturation in other proteins, and interpret them tentatively in terms of changes in the configuration of the myosin molecule. Comparison with the data of Paper I also reveals very great differences between the effects of the reagents studied on —SH groups, on the one hand, and their effects on viscosity and double refraction of flow, on the other.

### EXPERIMENTAL

The preparation of rabbit myosin has been described in Paper I.

*Lobster myosin* was prepared from the claw and tail muscle of lobsters by an essentially identical process. It was found that

lobster myosin could not be concentrated as effectively as rabbit myosin by centrifuging. The solutions of lobster myosin obtained were all below 1 per cent protein concentration, but they were somewhat more viscous than rabbit myosin at the same concentration and showed very intense double refraction even at a concentration of 0.1 per cent. Qualitatively, however, these two types of myosin were very similar in all their properties.

*Double refraction of flow* was conveniently observed by a simple arrangement consisting of two Polaroid disks, one above the other, illuminated by a light passing upwards through a hole in a small bench or table.<sup>1</sup> The Polaroid disks were set so that the illumination was at a minimum (Polaroid is not a perfect polarizer and always transmits a small amount of red and violet light). The protein solution to be studied was then placed in a small beaker between the two Polaroid disks, and stirred with a black painted glass rod. When the myosin solution was at rest, the field remained dark, but it immediately became bright on being stirred. If the stirring was done with a rotary motion, the appearance of a dark cross of isocline on the bright field could readily be observed in solutions of moderate concentration. In a solution containing 1 per cent of undenatured rabbit myosin, double refraction was readily observed by this means when the depth of the solution in the beaker was only 3 or 4 mm. In a solution containing 0.5 per cent myosin, double refraction was easily seen in a layer 1 or 2 cm. in depth. The addition of any reagent which destroys double refraction of flow caused a steady decline in the brightness of the stirred solution, and a gradual fading of the cross of isocline until it merged with the background, and the whole field became uniformly dark. The time taken for this process to occur varied greatly with the concentration and nature of the denaturing agent, and could be estimated reproducibly with an error of about 10 per cent. The measurements reported in this paper give a preliminary survey of the behavior of a wide variety of compounds.

<sup>1</sup> The quantitative measurement of the angle of isocline and double refraction of flow has already been described (18, 19). A portable apparatus, which permits rapid, roughly quantitative measurements on solutions of highly anisotropic molecules, has been developed by the authors of this paper and will be reported later.

A quantitative study of some of the more important of these will be reported later.

*Viscosity*—Viscosity was determined in capillary viscometers of the type employed by Bingham and Jackson (4) in which the liquid can be made to flow through the capillary in either direction. The variable hydrostatic pressure used in forcing the liquid through the capillary was produced by a compressed air system with an adjustable side outlet allowing the excess air to bubble out under water. By raising or lowering the side tube in the water, the pressure could be varied. Low hydrostatic pressures were read by means of a water manometer; higher pressures, by means of a mercury manometer. The times of outflow could in general be determined to considerably better than 1 per cent. The errors in the hydrostatic pressure readings may, however, be as great as 2 per cent.

The volume of liquid which flowed through the viscometer employed, in the course of any measurement, was 4.0 cc.; the radius of the capillary (calculated by Poiseuille's law from the rate of flow of water through it at 25°, and the length of the capillary) was 0.042 cm. These data are of importance in evaluating the mean velocity gradient in the flowing liquid, which is of importance in the interpretation of the measured viscosities at different rates of flow.

### *Results*

#### *Experiments upon Double Refraction of Flow of Myosin*

*Effect of Salts, Dipolar Ions, and Urea*—Von Muralt and Edsall (19) noted that urea, iodides, and thiocyanates rapidly diminished double refraction of flow in myosin, to such an extent that the solution appeared isotropic in the apparatus employed. Further investigation has shown that many other compounds, including some not commonly regarded as denaturing agents, produce the same effect. The phenomenon is readily observed with the simple apparatus described in this paper. At the same time it is easy to observe from the motion of small particles suspended in the liquid that the viscosity has diminished, as is shown in detail by the quantitative measurements reported later in this paper.

The effects of most substances on rabbit and on lobster myosin

are very nearly the same. In general, lobster myosin is more strongly birefringent at a given concentration than rabbit myosin; it is appreciably thixotropic, and behaves like an elastic solid when subjected to small shearing stresses. Rabbit myosin, on the other hand, appears to behave as a highly viscous liquid when dissolved in salt, although in the absence of salt it readily forms a thixotropic gel (7). Both proteins, however, lose their double refraction in the presence of the same reagents, acting at approximately the same concentration. Lobster myosin appears to be somewhat more sensitive to denaturing agents.

The effect of a large number of reagents on double refraction of flow is shown in Table I.

The number and diversity of substances which destroy double refraction are noteworthy. The effect is produced, indeed, by almost any electrolyte in sufficient concentration: thus in 2 M potassium chloride, a marked diminution in double refraction is found within 2 hours, and its disappearance follows within 48 hours, although in 0.5 M potassium chloride myosin shows intense double refraction for an almost indefinite period.

The data reveal certain definite correlations between chemical structure and denaturing effect.

1. Salts containing the guanidine nucleus—the hydrochlorides of guanidine itself and of methylguanidine, and the monohydrochloride of arginine—destroy double refraction rapidly at low concentrations. A saturated solution of creatine (approximately 0.055 M) produces no loss of double refraction in myosin within a period of a week. N-Acetyl arginine destroys double refraction slowly at 0.45 M, over a period of hours. Thus dipolar ions containing the guanidine nucleus appear to be less powerful in this respect than simple ions. Urea and methylurea have an effect similar to that of guanidine salts, but only at concentrations about 5 times as great. All of these substances profoundly influence double refraction at concentrations too low to affect the titratable—SH content of myosin appreciably (12).

2. In terms of this criterion of denaturation, the chlorides of bivalent cations ( $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{Ba}^{++}$ ) are among the most powerful denaturing agents to be found, since they produce rapid changes in myosin at concentrations at or below 0.3 M.

3. The denaturing action of the halogens increases in the order

TABLE I

*Disappearance of Double Refraction of Flow in Myosin Solutions*

The column headed "Time" indicates the time elapsing before double refraction of flow became unobservable by the technique employed in these studies, after the indicated reagent at the indicated concentration had been added. All reagents were added to myosin dissolved in approximately 0.5 M KCl, pH between 6.2 and 7.4, at room temperature or below. (The temperature coefficient of the reactions involved was not large.) Control tests showed that variations of pH between 6 and 8 had no appreciable influence on the velocity of the loss of double refraction.

Reagent concentrations are given in moles per liter of myosin solution.

Reagent	Rabbit myosin			Lobster myosin		
	Concentration of reagent	Time	Preparation No.	Concentration of reagent	Time	Preparation No.
KCl.....	0.5	2 wks.	All	0.5	3 wks.	I, II, III
" .....	2.25	40 hrs.	V	1.75	10 min.	"
KBr.....	0.44	20 min.	III			
" .....	0.80	5 "	" V			
KI.....	0.28	20-25 min.	"	0.44	5 min.	I
" .....				0.80	20 sec.	"
NaCl.....	1.9	1 hr.	V			
LiCl.....	0.7	5 min.	"			
" .....	0.56	20 "	VII			
" .....	1.0	15 "	"	1.0	10 min.	I
NH <sub>4</sub> Cl.....	1.4	15 "	III			
CH <sub>3</sub> NH <sub>3</sub> Cl.....	1.4	10 "	"			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	1.6	5 "	"			
K <sub>2</sub> SO <sub>4</sub> .....	0.5	1 wk.	"			
MgCl <sub>2</sub> .....	0.35	2 min.	"	0.25	5 min.	I
" .....	0.4	5 "	V	0.28	90 sec.	IIB
" .....				0.53	5 "	"
MgSO <sub>4</sub> .....				0.35	8 min.	"
" .....				0.62	3 "	"
CaCl <sub>2</sub> .....	0.27	10 min.	V	0.11	7 "	II
" .....	0.54	<10 sec.	"	0.22	30 sec.	"
BaCl <sub>2</sub> .....	1.1	<10 "	"	0.30	10 min.	I
" .....				0.50	2 "	"
KSCN.....				0.10	30 "	"
" .....				0.19	7 "	"
" .....	0.30	<10 sec.	III	0.28	90 sec.	"
" .....				0.50	<5 "	"



TABLE I—*Concluded*

Reagent	Rabbit myosin			Lobster myosin		
	Concentration of reagent	Time	Preparation No.	Concentration of reagent	Time	Preparation No.
Glycine.....	1.7	>1 wk.	III	1.0	20 min.	I
Urea.....				0.43	>48 hrs.	"
" .....				0.88	30 min.	"
" .....	1.3	10-15 min.	I	1.1	20 "	"
" .....	2.0	<2 min.	"	1.6	12 "	"
N-Methylurea...	1.7	2 "	III			
Guanidine HCl	0.24	40 "	IX	0.24	12 min.	I
" " ..	0.27	13 "	"			
" " ..	0.32	6 "	"			
" HBr..	0.20	30 "	VII			
" " ..	0.33	4 "	"	0.33	<2 min.	I
" HI...	0.075	>45 hrs.	"			
" " ..	0.14	1 hr.	"			
" " ..	0.28	30 sec.	"			
" H <sub>2</sub> SO <sub>4</sub>	0.3	2 min.	V			
Methylguanidine						
HCl.....	0.21	25 "	III			
" " ..	0.45	2 "	"			
Arginine mono-						
hydrochloride..	0.36	2 "	"			
" " ..	0.26	>15 min.	V			
" " ..	0.35	12 "	"			
Creatine.....	0.055	>4 days	III			
N-Acetyl argi-						
nine.....	0.45	2-5 hrs.	VII			
Glycerol.....				1.6	20 sec.	I

Solutions of rabbit myosin were very stable in  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  buffers (pH 7, ionic strength 0.5), but lost double refraction slowly, over a period of days, in phosphate buffers at ionic strengths above 1.0.

$\text{Cl}^- < \text{Br}^- < \text{I}^-$ ; that of the cations of the alkali metals in the order  $\text{K}^+ < \text{Na}^+ < \text{Li}^+$ .<sup>2</sup>

<sup>2</sup> Some experiments were run with rubidium and cesium chlorides at concentrations of 0.7 to 1.0 M. Rabbit myosin lost its double refraction of flow within an hour in these solutions. The purity of the salts employed, however, was not certain, and the results are to be regarded as only tentative.

4. Ammonium chloride and its methylated derivatives produced rapid loss of double refraction at concentrations near 1.4 M;<sup>3</sup> but glycine, although containing an  $-\text{NH}_3^+$  group, had no effect on rabbit myosin at 1.7 M, even in the presence of considerable concentrations of chloride ion. Thus the disappearance of  $-\text{SH}$  groups in the presence of ammonium derivatives (12) is not necessarily associated with loss of double refraction.

5. Sulfates appear to have less denaturing action than the corresponding chlorides. Thus ammonium sulfate, at a given pH and  $\text{NH}_4^+$  ion concentration, has much less denaturing action than ammonium chloride; and magnesium sulfate produces rapid denaturation only at a distinctly higher molar concentration than magnesium chloride.

6. The complete oxidation of native myosin with porphyrindin does not impair double refraction of flow.

7. As judged by the criterion of preserving double refraction of flow, myosin is most stable in potassium chloride, potassium sulfate, and potassium phosphate buffers near pH 7. Myosin appears to be somewhat less stable in the corresponding sodium salts (see Mirsky (17)).

8. Concentration of denaturing agents had an extraordinarily great effect on the velocity of loss of double refraction. Thus (Table I) rabbit myosin was stable almost indefinitely in guanidonium iodide at 0.075 M. At 0.14 M, double refraction disappeared within 1 hour; at 0.28 M, within 30 seconds. The great changes in velocity of the process with small changes in guanidine hydrochloride concentration are quantitatively illustrated in Fig. 1. The logarithm of the double refraction is approximately a linear function of the time in these curves; but nothing as to the mechanism of the process is to be inferred from this fact. Quantitative study of the velocity and mechanism of the processes involved is now being undertaken on an extensive scale.<sup>4</sup>

<sup>3</sup> In working with solutions of ammonium salts, it is important that the pH should be adjusted to a value near 7. The pH was always tested, and adjusted if necessary, in the experiments reported here.

<sup>4</sup> Inspection of the data in Table I shows that the velocity of loss of double refraction, for the same reagent at the same concentration, varied somewhat from one myosin preparation to another, although the relative effects of different reagents remained essentially the same. The quantita-

*Effect of Alkaline Solutions*—Rabbit myosin was found to be very stable at pH values below 9.7, in buffer solutions of glycine and potassium glycinate (the total concentration of glycine and glycinate was 0.4 M). At pH 10.28 double refraction of myosin was partly gone in 5 minutes and completely gone after the solution had stood 1 hour in the cold. When glycinate buffer at pH 10.65 was added to rabbit myosin, double refraction diminished greatly at once and was completely gone in 5 minutes. These

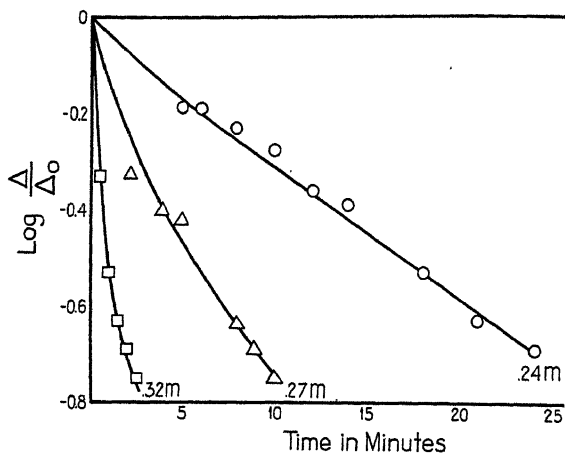


FIG. 1. Decrease of double refraction in rabbit myosin, dissolved in KCl, in the presence of guanidine hydrochloride, 0.24, 0.27, and 0.32 M; temperature 25°; pH 6.4. Abscissa, time in minutes; ordinate,  $\log \Delta/\Delta_0$ , where  $\Delta_0$  is the magnitude of double refraction at time 0, and  $\Delta$  is the double refraction after time  $t$ . (Measurements by H. O. Singher.)

data confirm the position of the alkaline pH stability range found by von Muralto and Edsall (19), who used a somewhat cruder chemical treatment.

tive studies now in progress may throw further light on this phenomenon. It appeared possible that some of the observed denaturing effects were due to small and variable amounts of impurities in the reagents used. The effects of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{MgSO}_4$ , however, were found to be the same after three recrystallizations as when the original c.p. salts were used. Thus, no evidence appeared for attributing the observed effects to impurities.

Lobster myosin lost its double refraction of flow rapidly at pH 10.3, and more slowly even at pH 9. Rabbit myosin is quite stable at the latter pH value.

*Effect of Acids*—Between pH 5 and 6 myosin is insoluble at all salt concentrations. When sufficient acid is added to make the pH somewhat more acid than 5, in the absence of excess salt, the myosin redissolves. The condition of the protein in this acid zone, however, is very different from its undenatured state in neutral solutions; it has lost its double refraction of flow, and is now readily precipitated by the addition of even small amounts of neutral salts. If such a solution is again brought to a pH near 6, it again goes into solution on the addition of salt, but we have not observed restoration of double refraction of flow under these conditions. The detailed protocol of one such experiment follows.

15 cc. of rabbit Myosin IIb (containing 1 per cent protein in 0.5 N KCl at pH 6.2, and showing strong double refraction) were diluted with 80 cc. of cold distilled water and centrifuged in the cold room. The precipitate packed very tightly; it was again twice washed with 80 cc. of cold distilled water and centrifuged each time. The final precipitate was dissolved in 0.6 cc. of N/7 HCl. It formed a clear solution, with a few disk-shaped particles of undissolved myosin floating in it. The pH of the solution was 4.3, by the glass electrode. The solution showed no double refraction of flow, although the disk-like particles in it were strongly doubly refractive (optic axis generally parallel to the long axis of the disk).

About 1 cc. of this solution was set aside for pH determination; the rest was treated with 0.86 cc. of 0.1 N NaOH, with constant stirring. The protein set to a clear gel (*cf.* Edsall (7)) which flocculated on the addition of a small amount of KCl, and the precipitate then dissolved to form a clear solution when more KCl was added. The pH of this solution was 5.7; it showed no trace of double refraction of flow.

Experiments of this type were carried out on three different rabbit myosin preparations, with the same results. Thus we do not confirm the statement of Bate Smith (3) that acid-treated myosin would again become doubly refractive on being dissolved in solutions at pH above 6. Neither have we been able to confirm the statement of von Muralt and Edsall (19), that myosin at pH 4.6 shows double refraction of flow.

*Heat Denaturation of Lobster Myosin*—The effect of heat on frog and rabbit myosin has been described in detail by Mirsky (17); these myosins begin to coagulate rapidly near 38°, and the ob-

servations on the effect of heating rabbit myosin, made by us, agree essentially with those of Mirsky. Lobster myosin at a concentration of 1 per cent also precipitates rapidly when it is heated near 100° for 2 or 3 minutes; at lower concentrations, however (below 0.4 per cent), it does not precipitate, even when heated in a boiling water bath for 6 minutes. Instead, the solution becomes distinctly more opalescent and much less viscous (after cooling) than it originally was. Double refraction of flow is completely destroyed. The protein still precipitates when brought by acid to a pH between 5 and 6, but the precipitate is fine and flocculent, not stringy and gelatinous like the acid precipitate of native myosin.

Lobster myosin heated 10 minutes to 40° loses almost all its double refraction, and becomes much less viscous than before.

#### *Studies on Viscosity of Myosin Solutions*

*Theoretical Considerations*—Because of its extreme molecular asymmetry, myosin gives very viscous solutions. The close relationship between the shape of molecules and the viscosity of their solutions is now abundantly clear from the work of several investigators (6, 9, 13, 22), although agreement has not yet been reached as to the exact nature of the functional relation involved. In any case, the experimental data are best formulated for theoretical discussion in terms of the quantity  $\nu$ , the ratio of specific viscosity ( $\eta_{sp}$ ) to volume fraction ( $\Phi$ ) of solute.

$$\nu = \frac{\eta_{sp}}{\Phi} = \frac{1}{\Phi} \left( \frac{\eta}{\eta_0} - 1 \right) \quad (1)$$

( $\eta$  is the viscosity of the protein solution;  $\eta_0$  that of the solvent.) For a solute made up of incompressible uncharged spherical molecules,  $\nu$  should be 2.5 from Einstein's theory (8); for all non-spherical molecules it is greater than 2.5, owing to the additional work which must be done in rotating such molecules under the influence of the velocity gradient in the liquid. This work, and therefore the measured apparent viscosity, varies with the magnitude of the velocity gradient. When this gradient approaches 0, the orientation of the molecules is purely random, owing to their Brownian movement, and  $\nu$  (at constant  $\Phi$ ) approaches a maximal value  $\nu_0$ . At very high velocity gradients the molecules

are highly oriented by the shearing stresses in the liquid, and  $\nu$  tends asymptotically to a lower limiting value  $\nu_\infty$ . Hence, for the interpretation of viscosity measurements in molecules like myosin, the velocity gradient in the viscometer must be considered.

In capillary viscometers, the velocity gradient varies from 0 at the center of the capillary to a maximum value at the wall. According to Kroepelin (14), however, we may describe the measurements in terms of a "mean velocity gradient" ( $\bar{\beta}$ ) which Kroepelin has shown to be a function of the volume  $V$  of liquid flowing through the capillary in time  $t$ , and of the capillary radius  $R$ .

$$\bar{\beta} = \frac{8V}{3\pi R^3 t} \quad (2)$$

It has been found experimentally (14, 23) that the apparent viscosity is a unique function of  $\bar{\beta}$  for liquids showing anomalous viscosity, and is independent of the dimensions of the particular capillary used.<sup>5</sup>

Whether a measured value of  $\nu$  corresponds to  $\nu_0$ ,  $\nu_\infty$ , or some intermediate value depends on the ratio of the velocity gradient,  $\beta$ , to  $\Theta$ , the rotary diffusion constant (5, 29) of the protein. If  $\beta/\Theta < 1$ , the measured value will correspond to  $\nu_0$ ; if  $\beta/\Theta \gg 1$ , it will closely approach  $\nu_\infty$  (22). For nearly all the so called globular proteins—proteins for which the ratio of long to short axis is not greater than 7 : 1 or 8 : 1—the former case is realized; for the mean velocity gradients found in most capillary viscometers range from a few hundred to a few thousand  $\text{sec}^{-1}$ ; while the rotary diffusion constants found for such proteins in water at 25° vary from  $6 \times 10^6 \text{ sec}^{-1}$  for hemoglobin (21) to  $3 \times 10^6 \text{ sec}^{-1}$  for pseudoglobulin (10). Thus the apparent viscosities found for solutions of these proteins should not, and experimentally do not, vary appreciably with the velocity gradient; they correspond to measurements made under conditions of ran-

<sup>5</sup> It is true, as Philippoff (23) has pointed out, that, for liquids whose apparent viscosity varies with the rate of shear,  $\bar{\beta}$  is not a strictly linear function of the true mean velocity gradient in the liquid. It is, however, not far from equal to the mean gradient, and appears to be definitely the best function at present available for describing viscosity measurements on a capillary viscometer for substances such as myosin.

dom orientation of the molecules. This was for instance undoubtedly the case for the recent measurements of Neurath and Saum (20) on serum albumin.

*Experiments on Undenatured Myosin*—In the present myosin studies, the situation is very different. The rotary diffusion constant of myosin lies between 8 and 12 sec.<sup>-1</sup>, according to the calculations of Mehl (16) from the data of von Muralt and Edsall (18, 19). Therefore, the values of  $\beta/\theta$ , under the conditions of our measurements, lie between 40 and 200, and the myosin mole-

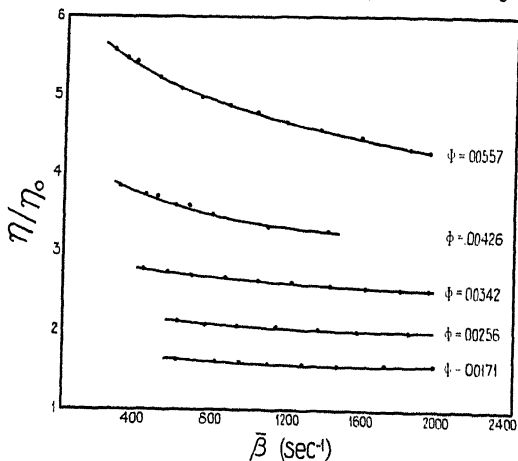


FIG. 2. Relative viscosity of rabbit myosin solutions, plotted as a function of the mean velocity gradient,  $\beta$  (see text). The volume fraction ( $\phi$ ) of myosin in each solution studied is indicated at the right of each curve. Temperature 25°.

cules are highly oriented in the liquid flowing through the capillary. In Fig. 2 the apparent specific viscosity of undenatured rabbit myosin solutions, dissolved in KCl, is shown as a function of the mean velocity gradient. The viscosity falls with increasing velocity gradient, and at least in the more dilute solutions appears to approach very closely a limiting lower value. This limiting value, divided by the volume fraction of myosin in the solution, may be taken as equivalent to  $\nu_\infty$ , as defined above. The calculated values of  $\nu_\infty$  for the solutions shown in Fig. 2 are given in Table II. The fact that these values vary with the concentration

shows that there is interaction between the myosin molecules at all the concentrations studied, since at sufficiently great dilutions  $\nu_{\infty}$  should be independent of  $\Phi$ . The extrapolated value,  $\nu_{\infty} = 240$  at 0 concentration, indicates the extreme asymmetry of the myosin molecules. The values recently given by Polson (24) for  $\nu_0$  for a number of other proteins range from 5.7 for egg albumin to 14.6 for gliadin. The value of  $\nu_0$  for myosin cannot be obtained from the present data—it could only be measured in a viscometer of the Couette type where measurements can be made at very low velocity gradients—but it must in any case be much higher than 240.<sup>6</sup>

TABLE II  
*Specific Viscosity of Rabbit Myosin in Dilute Solution at High Velocity Gradient*

Solvent, KCl 0.5 M; pH 6.2; temperature 25°.

Volume fraction of myosin ( $\Phi$ )	$\eta_{sp} = \frac{\eta}{\eta_0} - 1$	$\frac{\eta_{sp}}{\Phi} = \nu_{\infty}$
$4.26 \times 10^{-3}$	<2.26	<531
$3.42 \times 10^{-3}$	1.52	444
$2.56 \times 10^{-3}$	1.00	391
$1.71 \times 10^{-3}$	0.58	340
0 (Extrapolation)		240

The apparent specific volume of myosin is taken as 0.75 in calculating the volume fraction from the weight fraction.

Quantitatively, at present, it appears impossible to estimate the ratio of the long and short axes of the myosin molecule from these data. The equation of Eizenschitz (9), applicable to ellipsoids of revolution, is

$$\nu_{\infty} = \frac{1.15 a/b}{\pi \ln (2 a/b)} \quad (3)$$

<sup>6</sup> Some recent results on tobacco mosaic virus illustrate this point. Lauffer (15) found for a 0.0296 per cent virus solution in a capillary viscometer a value of  $\nu = 55.8$ . This value is probably close to  $\nu_{\infty}$ , although the exact velocity gradient was not given. Robinson (25), employing a Couette viscometer, found  $\nu_0 = 1500$  for a 0.02 per cent virus solution. These measurements were made on different preparations, and are therefore not strictly comparable, but they illustrate vividly the importance of considering the velocity gradient in viscosity measurements on highly asymmetrical molecules.



where  $a$  and  $b$  are the long and short axes, respectively, of the ellipsoid. Taking  $\nu_{\infty}$  at  $\Phi = 0$  as 240 for myosin gives a value of approximately 6000 for the ratio of the long to the short axis, which is impossibly high, since the length of the myosin molecule is of the order of 5000 to 10,000 Å. (16) and its cross-section cannot be less than 10 Å., and is probably greater. The recent, and apparently very exact treatment of Peterlin (22) does not include the evaluation of  $\nu_{\infty}$  for such very large axial ratios. It is clear from his figures, however, that the observed value of  $\nu_{\infty}$  should correspond to an extremely high axial ratio. Peterlin's treatment

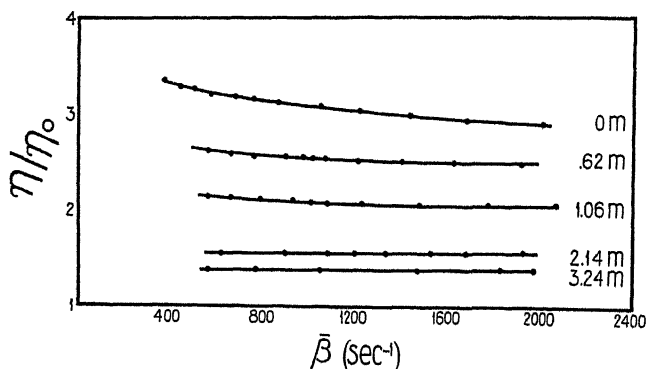


FIG. 3. Relative viscosity of rabbit myosin solutions as a function of the mean velocity gradient and concentration of guanidine hydrochloride. All solutions contained myosin, volume fraction  $\Phi = 0.00372$ , and KCl, 0.5 M. Top curve, no guanidine (undenatured myosin). Solutions shown in the lower four curves contained respectively 0.62, 1.06, 2.14, and 3.24 M guanidine hydrochloride. Temperature 25°.

is strictly applicable only to solutions of ellipsoids which are (1) rigid, (2) monodisperse, (3) uncharged, and in an indifferent (non-polar) solvent. It is certain that the last condition is not fulfilled by myosin solutions, and highly likely that the other two are not. At present, therefore, we may only draw the qualitative conclusion that the viscosity of undenatured myosin solutions gives evidence of the high asymmetry of the myosin molecules.

*Action of Denaturing Agents on Viscosity*—Reagents such as guanidine and urea diminish the viscosity of myosin solutions, and by inference, therefore, the asymmetry of the molecules also.

The variation of viscosity with concentration of guanidine hydrochloride, at constant myosin concentration, is represented in Fig. 3, and the corresponding fall in the values of  $\nu_{\infty}$  is recorded in Table III, for this and other denaturing agents.

The great decrease observed in guanidine hydrochloride, from  $\nu_{\infty} = 484$ , in KCl without guanidine, to  $\nu_{\infty} = 97$  in 3.24 M guanidine reflects a very marked decrease in the asymmetry of the molecule. As with all the other effects studied, urea is found to be less potent in its action, a 5 M solution of urea being approximately equivalent to a 3.24 M solution of guanidine hydrochloride. Glycine (0.1 M), which abolishes titratable —SH groups but does not destroy

TABLE III

*Effect of Denaturing Agents on Viscosity of Rabbit Myosin at High Velocity Gradient*

Myosin concentration,  $4.96 \times 10^{-3}$  gm. per cc. of solution =  $3.72 \times 10^{-3}$  cc. per cc. of solution.

Substance added to 0.5 M KCl in solvent	$\eta_{sp}$	$\nu_{\infty}$
None.....	1.80	484
0.10 M glycine.....	1.80	484
1.5 " $\text{NH}_4\text{Cl}$ .....	1.45	390
0.62 " guanidine HCl.....	1.40	376
1.06 " " ".....	1.04	279
2.14 " " ".....	0.54	145
3.24 " " ".....	0.36	97
2.5 " urea.....	0.84	226
5.0 " ".....	0.34	91

double refraction, has no effect on the viscosity. On the other hand, 1.5 M ammonium chloride, which abolishes titratable —SH and also destroys double refraction, lowers the viscosity markedly. *There appears to be an invariable correlation between diminution in viscosity of myosin solutions and loss of double refraction, while there is no correlation between viscosity changes and alteration in —SH groups.*

The effect of denaturing agents on myosin is in pronounced contrast to their effect on the "globular" proteins, whose viscosity increases in the presence of denaturing agents, such as urea (see Anson and Mirsky (1)). Thus the data of Neurath and Saum

(20) show that  $\nu$  for serum albumin increases from 6.50 in water to 22.6 in 6.66 M urea, figures which offer a striking contrast to the data in Table III, whereas the measurements of Frampton and Saum (11) on tobacco mosaic virus indicate that urea has an effect on it qualitatively similar to its effect on myosin.

The effect of denaturing agents on viscosity is progressive; the higher the concentration of guanidine or urea, the lower the level to which the viscosity falls. Even the lowest concentrations employed in our viscosity studies, however, are sufficient to reduce double refraction of flow below the point where it can be detected by the apparatus described in this paper. Further changes which take place at higher concentrations of guanidine or urea are, however, revealed by viscosity measurements. An apparatus capable of producing very high velocity gradients, now being constructed, should also reveal changes in double refraction of flow, and such measurements will subsequently be reported.

*Solubility of Native and Denatured Myosin*—None of the denaturing agents employed in these studies (except in some cases heat) destroys the solubility of myosin in ordinary salt solutions. After treatment with any one of them, the protein may be precipitated by dilution with cold distilled water, centrifuged, and washed repeatedly until it is free of the denaturing agent, and then redissolved by addition of potassium chloride. It continues to behave like a typical globulin; if anything, it is perhaps more readily soluble in dilute salt solutions than before denaturation. No treatment yet employed by us, however, has ever restored double refraction of flow when this property has once been lost. It cannot be positively stated that this change is irreversible, but up to the present it has certainly not been reversed.

#### DISCUSSION

*Lack of Correlation between Changes in Titratable —SH Groups and in Double Refraction of Flow*—The changes in myosin revealed by the porphyrindin titration (12) appear to be entirely different from those revealed by the methods described in this paper. It is true that urea and guanidine hydrochloride increase titratable —SH groups and also destroy double refraction of flow. But the latter effect occurs at relatively low, the former only at much

higher, concentrations of the denaturing agent. Thus 0.27 M guanidine hydrochloride diminishes double refraction of flow in 13 minutes below the point where it can be detected (see Table I); but no appreciable change in titratable —SH groups can be detected unless the guanidine is about 1 M (see Table II, Paper I), and far higher concentrations of guanidine are necessary to release the maximal number of titratable —SH groups. Similar relations hold for urea, although its effect both on double refraction and on —SH groups is weaker than that of guanidine hydrochloride.

The effect of other denaturing agents reveals even more striking differences. Ammonium salts and glycine reduce titratable —SH groups in myosin to 0; ammonium salts destroy double refraction of flow, while glycine does not. Salts such as KI, KSCN,  $MgCl_2$ ,  $CaCl_2$ , and LiCl have no effect on titratable —SH groups, but are very powerful reagents for destroying double refraction of flow. Thus substances which destroy double refraction of flow may increase titratable sulfhydryl groups, may abolish them, or may leave them unchanged. On the other hand, decrease of double refraction of flow appears always to go hand in hand with decrease in viscosity of myosin, whether the change be produced by chemical agents or by heat. Changes in both of these properties reflect changes in molecular asymmetry, whereas changes in —SH groups depend on other alterations in molecular configuration, whose nature is at present less clearly defined.

*Relation of These Data to Ultracentrifugal Studies*—The effect of denaturing agents on viscosity and double refraction, reported in this paper, shows unmistakably that the length and asymmetry of the molecule are reduced by such reagents. It may be inferred that the molecular weight is also reduced in such solvents; and in the case of urea there is direct evidence for such a change from the osmotic pressure measurements of Weber and Stöver (28). These authors found the mean molecular weight of undenatured myosin to be of the order of 1,000,000, while in concentrated urea solution it was of the order of 100,000.

The loss of the double refraction of myosin in solutions of denaturing agents may be comparable to the dissociation of other proteins observed in ultracentrifugal studies. The dissociation of the hemocyanins, for instance, is particularly affected by such reagents as guanidine salts and divalent cations (27), which are

also particularly effective in destroying the double refraction of myosin. The underlying basis of the observed effects on myosin, then, is probably a mechanism common to many, if not all, other proteins. The method of double refraction of flow, however, when applicable, offers a particularly simple technique for revealing such changes.

*Hofmeister Series*—There appears to be a certain correlation between the effect of salts on double refraction of myosin and their position in the Hofmeister series. Salts which are effective salting-out agents, such as sulfates and phosphates, have little effect in destroying double refraction; while salts of lithium, of ammonium, and of the divalent cations have little salting-out action and destroy double refraction rapidly. We shall make no attempt in this study, however, to propose a theory for the mechanism of the observed effects.

*Comparison with Tobacco Mosaic Virus*—It should not be inferred that the effects observed for myosin will be found in all other rod-shaped proteins. Preliminary studies of the effect of divalent cations on tobacco mosaic virus solutions<sup>7</sup> have shown changes profoundly different from those observed in myosin. Calcium, magnesium, or barium chloride (0.1 to 0.5 M) added to tobacco virus in water produce a temporary decrease in double refraction of flow followed by a rise to or above the initial value. The "relaxation time" of the molecules also increases, as judged by the time taken for double refraction to disappear after stirring of the liquid ceases. In the course of half an hour the liquid becomes cloudy and develops an opalescent crystalline sheen; within a few days a precipitate slowly settles out. The entire course of the reaction indicates that an aggregation of the original protein molecules is taking place; not a dissociation, as in the case of myosin.

Urea slowly destroys the double refraction of flow in tobacco virus, but even in 6 M urea, as the recent work of Stanley and Lauffer (26) shows, double refraction falls to only one-third of the original level in 2 hours; while 1.5 M urea destroys the double

<sup>7</sup> We are indebted to Dr. W. M. Stanley and Dr. M. A. Lauffer for providing us with these preparations of tobacco mosaic virus. The studies made upon them in this laboratory have in part been already reported (16), and a more complete account of them will be given later.

refraction of myosin far more completely within 5 minutes. Thus the extreme lability characteristic of myosin is not necessarily at all characteristic of other asymmetric proteins.

*Significance of Viscosity Changes*—The increase of viscosity on denaturation in such proteins as egg albumin and edestin has been interpreted by Astbury, Dickinson, and Bailey (2) as due to the unfolding of the compact undenatured protein into a more extended polypeptide chain. The decrease in viscosity of myosin, arising from the action of the same denaturing agents that increase the viscosity of "globular" proteins, is interpreted by us as due to the breaking up of the very elongated native myosin into smaller and less asymmetrical molecules. Obviously there is no contradiction between the two sets of phenomena. Presumably, in either case, certain linkages within the molecule are broken, and these linkages may well be the same in all these different proteins. *The changes in viscosity depend, not on the nature of the linkages broken, but on the geometrical configuration of the undenatured molecule and on the changes in that configuration brought about by the denaturing agent.*

*Concerning the Preparation of Myosin*—The observations recorded in this paper indicate the importance of the solvent used in extracting myosin from muscle. Potassium chloride, phosphate, and sulfate appear to be by far the most desirable salts to employ if undenatured, doubly refractive myosin is to be obtained. Salts such as lithium and ammonium chlorides, which have often been employed as solvents for extraction, will almost certainly yield an altered product. Also, the great effect of moderate rise of temperature in diminishing double refraction emphasizes the desirability of carrying out the preparation and of keeping the purified myosin, as far as possible, in the cold. In any case, it is desirable that the protein should be tested for double refraction each time after it has been precipitated and redissolved. Even with all the precautions indicated in this paper, occasional preparations have been found to have lost most of their double refraction.

It is possible that the changes in myosin here reported have some biological significance in connection with muscular activity. The fact that the loss of double refraction occurs with such ease, and is readily produced by substances present in the muscle fiber, such

as guanidine derivatives and magnesium ions, makes this a tempting speculation. The apparent irreversibility of the process is against this view, however, and it scarcely calls for further discussion until more evidence is brought forward.

We are indebted to Mr. H. O. Singher for much valuable work in the preparation of the myosin used in these studies.

#### SUMMARY

1. Double refraction of flow in myosin is profoundly and rapidly diminished by a large variety of organic and inorganic substances, acting at relatively low concentrations. Among the most powerful of these substances are guanidine and methylguanidine salts, arginine monohydrochloride, calcium and magnesium chlorides, potassium iodide, and thiocyanate, all of which produce well marked effects at concentrations below 0.3 M. Their effects on rabbit and on lobster myosin are nearly identical.

2. Lithium and ammonium chlorides, urea, and potassium bromide produce similar effects at somewhat higher concentrations. Sulfates have less denaturing action than the corresponding chlorides when the comparison is made at the same cation concentration.

3. Myosin is most stable in the cold near pH 7, dissolved in potassium chloride, phosphate, or sulfate. Rabbit myosin, dissolved in potassium chloride, is very stable in the presence of high concentrations of glycine; but molar glycine destroys double refraction in lobster myosin.

4. The effects of pH and of heat on double refraction of myosin are described.

5. The solubility of myosin is not significantly altered by the denaturing agents studied; it remains a typical globulin after double refraction is lost. So far the loss of double refraction has not been reversed.

6. The basis for the interpretation of viscosity measurements on solutions of large and very asymmetrical molecules is critically discussed. The viscosity of myosin decreases in the presence of all the denaturing agents studied. In conjunction with the double refraction measurements, this indicates a great decrease in the asymmetry of the molecule.

7. Effects of denaturing agents on viscosity are closely correlated with their effects on double refraction of flow. There is no correlation, however, between changes in either of these properties and changes in titratable sulfhydryl groups.

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## CANINE CYSTINURIA

### V. FAMILY HISTORY OF TWO CYSTINURIC IRISH TERRIERS AND CYSTINE DETERMINATIONS IN DOG URINE\*

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In Paper II of this series (1), the occurrence of cystinuria in two male Irish terriers was reported. The genetic relationship of these animals is given in Chart I. The original cystinuric dog is designated as Dog 1, the second cystinuric dog as Dog 4.

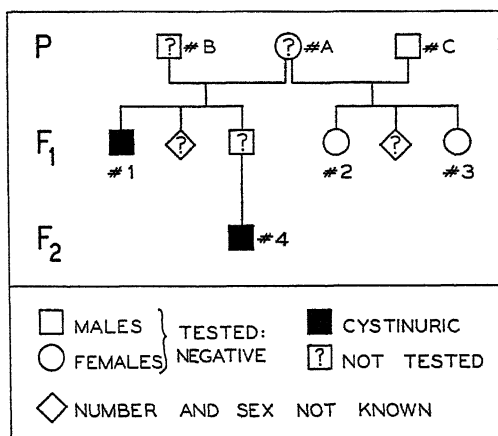


CHART I. Pedigree chart. Cystinuria in Irish terriers

Through the records of the American Kennel Club, it was possible to locate the owners of the sire (Dog B) and of the dam (Dog A) of Dog 1. There was no history of calculus formation or difficulty in urination with Dogs A or B, both of which were

\* Aided by a grant from the Rockefeller Foundation.

dead at the time of inquiry. The litter to which Dog 1 belonged was apparently quite normal and perfectly healthy. Subsequently the dam (Dog A) had been bred with an Irish terrier, Dog C, and had given birth to a litter. Dog C did not have cystinuria, as indicated by a negative cyanide-nitroprusside test in its urine. Two females from this litter (Dogs 2 and 3 in Chart I) were purchased and brought to the laboratory. The sulfur partition of their urine (reported in Table I together with that of a dog of another breed) is normal<sup>1</sup> and shows that they are not cystinuric.

A short time later, Dog 4 was placed in the Raritan Hospital for Animals for miscellaneous complaints, and it was found to be

TABLE I

*Sulfur Distribution in Urine of Two Normal Female Irish Terriers*

Dog No.	Per cent of total S					Per cent of neutral S	
	Inorganic sulfate	Ethereal sulfate	Total neutral S	Cystine S*	Undetermined neutral S	Cystine S*	Undetermined neutral S
2†	67	6	27	0.4	26	1.4	99
3‡	70	9	22	0.4	21	1.7	98
M§	62	7	31	2.0	29	7	93

\* Cuprous chloride precipitation.

† Total S = 620 mg. per liter.

‡ Total S = 600 mg. per liter.

§ Dog M, a female Dalmatian coach hound, is included for comparison; total S = 167 mg. per liter.

suffering from cystinuria (*cf.* (1)). Subsequent to this diagnosis, examination of its pedigree revealed that it was the son of a male litter mate of Dog 1. Through the courtesy of Dr. M. L. Morris, Dog 4 then came into our permanent possession, which made more detailed observations possible. However, metabolic experimentation had to be restricted, since this dog was of primary im-

<sup>1</sup> The neutral sulfur content of dog urine is higher than that of human urine, and may amount to from 10 to 60 per cent of the total sulfur. Under average conditions, dogs excrete about 30 per cent of the total sulfur as neutral sulfur. For instance, a dog weighing 9 kilos, fed Cowgill's diet corresponding to 4.8 gm. of nitrogen per day, excreted 30 to 34 per cent of the total urinary sulfur as neutral sulfur (2).

portance as a breeder. No catheterization for the collection of urine specimens was attempted.

For a period of about 2 months, Dog 4 was kept in a metabolism cage on a constant food intake, on which it maintained its weight. Unfortunately it then developed a diarrhea and had to be taken off this diet and returned to the kennel.

During the time of study, the urine was analyzed daily for total nitrogen, creatine, creatinine, and various sulfur constituents. It was found that the average excretion of 4 successive days gave representative values for the daily excretion. A typical set of

TABLE II

*Average Daily Excretion of Various Constituents in Urine of Dog 4*

Diet, modified Cowgill diet (3); daily food intake, 110 gm.; weight of dog, 11.4 kilos.

24 hr. excretion* of		Per cent of total S	Per cent of neutral S
	gm.		
Cystine (photometric (2))	0.17		
Total N	4.5		
Creatinine	0.33		
Creatine	0.14		
Total S	0.348		
Inorganic sulfate S	0.201	58	
Ethereal " "	0.035	10	
Total neutral S	0.112	32	
Cystine S	0.045	13	40
Undetermined neutral S	0.067	19	60

\* Average of 4 consecutive days.

figures is reported in Table II. On this dietary intake, Dog 4 excreted 170 mg. of cystine, or 15 mg. per kilo per day. Cystine sulfur accounted for 13 per cent of the total sulfur and 40 per cent of the neutral sulfur.

In carrying out cystine determinations with the urine of Dog 4, several difficulties were encountered. In the fresh urine, there was an unknown reducing substance<sup>2</sup> which interfered with the

<sup>2</sup> The interfering substance resembled ascorbic acid in its behavior in the photometric determination (4), inasmuch as the reduction of phosphotungstic acid was inhibited by mercuric chloride, but only partially

photometric (4) and Sullivan ((5), *cf.* (6)) methods. The effect of this interfering substance gradually disappeared on standing. After the urine had been aged for 1 to 3 days, reliable results could be obtained by the photometric method but the standing only partially eliminated the interference with the Sullivan reaction.

If the cystine in the urine was precipitated with cuprous chloride according to the method of Rossouw and Wilken-Jorden ((7), *cf.* (4)), the supernatant fluid (from which the copper had been removed by hydrogen sulfide and the latter by nitrogen) contained the interfering substance. When added in sufficient quantity to a cystine standard, the supernatant fluid completely inhibited color development in the Sullivan reaction.

The cuprous chloride method had originally been designed for protein hydrolysates, and to obtain accurate results with dog urine, a number of modifications had to be introduced. After considerable experimentation, the following procedure was adopted, in which the copper was removed in two steps, permitting cystine determinations by both the photometric and Sullivan methods.

To 35 cc. of urine in a graduated centrifuge tube, 2.5 cc. of glacial acetic acid are added and the mixture adjusted to pH 4.5 with concentrated potassium hydroxide (60 per cent). To this mixture is added 1 cc. of a special cuprous chloride solution (containing 0.2 per cent hydrochloric acid and 25 per cent potassium chloride and prepared as described (7)). The cuprous chloride solution is added, with constant stirring, slowly drop by drop over a period of at least 2 minutes. After 40 minutes at room temperature, the precipitate is centrifuged off, washed with 95 per cent alcohol, and dissolved in 5 cc. of 25 per cent potassium chloride in 1 per cent hydrochloric acid (traces of insoluble material may remain). After dilution to 12 to 13 cc. with water, 1 cc. of 10 per cent potassium thiocyanate is added, the volume adjusted to 15 cc., and the solution filtered from the copper thiocyanate which precipitates immediately and leaves only

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inhibited by mercuric chloride in the presence of sulfite. However, phosphotungstic acid is reduced by ascorbic acid in the presence of a mixture of cadmium and mercuric chlorides, while the urines containing the interfering substance did not develop color under these conditions.

traces of copper in solution. The filtrate is then used directly for the photometric cystine determination. To remove the last trace of copper, 5 cc. of the filtrate are treated in a 10 cc. graduate with 1 cc. of 5 per cent acetic acid and 0.5 cc. of 10 per cent potassium thiocyanate. The pH is adjusted to 4.5 with a few drops of pyridine and the volume made to 7.5 cc. To 4.5 cc. of the filtrate, 0.5 cc. of 10 per cent potassium hydroxide is added and the Sullivan reaction carried out in the regular manner ((5), *cf.* (6)).

Representative data are presented in Table III. The values for cystine obtained after cuprous chloride precipitation averaged

TABLE III  
*Cystine Determinations in Urine of Dog 4 by Various Methods*

Date	Direct determination		Cu <sub>2</sub> Cl <sub>2</sub> precipitation			
	Photo- metric	Sullivan	Photometric		Sullivan	
				Recovery		Recovery
<i>Apr., 1936</i>	<i>mg. per 24 hrs.</i>		<i>mg. per 24 hrs.</i>	<i>per cent</i>	<i>mg. per 24 hrs.</i>	<i>per cent</i>
3	90	Negative	79	87	84	93
4	180	Slightly +	149	83	160	89
5	182	Negative*	168	92	164	90
6	228	Slightly +	214	94	162	70
Average of 9 determinations† . . .				90		84

\* 4 days later the modified procedure (Sullivan Method B (9, 10)) gave 153 mg. of cystine in this specimen. (We are indebted to Dr. M. X. Sullivan for this determination.)

† The average includes five other determinations omitted from the table.

90 and 84 per cent by the photometric and Sullivan methods, respectively, of the cystine present, as estimated directly in aged urine by the photometric method.

That there are present in normal and cystinuric human urine substances which interfere with the Sullivan reaction is well known (5, 8). The inhibition with normal and cystinuric dog urine is much more marked. In order to overcome the effect of these urinary inhibitors, certain changes in the procedure were suggested by Sullivan<sup>3</sup> and Hess (9). This modification of the Sullivan

<sup>3</sup> We are indebted to Dr. M. X. Sullivan for making this method available before publication.

method has been extensively used by us for urine analysis (but is not recommended for protein hydrolysates; *cf.* (10)). With dog urines it gives 80 to 90 per cent of the photometric cystine values (*cf.* Table III; also (6, 10) where this method is designated as Sullivan Method B).

With some cystinuric dog urines, difficulties were encountered in obtaining positive cyanide-nitroprusside tests. This is due to the presence of interfering substances and can be overcome to some extent by use of larger amounts of nitroprusside.

#### SUMMARY

1. Further investigations on the occurrence of cystinuria in a family of Irish terriers are reported.

2. The cystine excretion and sulfur distribution in the urine of normal and cystinuric members of this family of dogs are presented.

3. Cystinuric dog urines frequently contain substances which interfere with the Folin, Sullivan, and nitroprusside reactions for cystine. Satisfactory conditions for cystine determinations have been established.

4. A procedure for the determination of cystine by both the photometric and Sullivan methods after cuprous chloride precipitation is described.

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# THE DETERMINATION OF REDUCING GROUPS WITH PORPHYRINDIN, WITH SPECIAL REFERENCE TO EGG ALBUMIN\*

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Kuhn and Desnuelle (1) have introduced the use of the blue dye, porphyrindin, for the determination of sulfhydryl groups in proteins. This dye has a very high potential (+0.57 volt at pH 7, referred to the normal hydrogen electrode (2)), and oxidizes cysteine stoichiometrically to cystine (1). They determined the —SH groups in heat-denatured egg albumin by adding increasing amounts of porphyrindin at 0° and pH 7.2 to a series of Thunberg tubes containing the same amount of protein, and noted in which the dye was completely decolorized. The —SH groups corresponded to 0.58 per cent cysteine (Greenstein (3) found 0.50 per cent by this method). In repeating Kuhn's experiments, we were not able to obtain a satisfactory end-point. There was a fairly clear demarcation between the colorless tubes and the first incompletely decolorized one, but in the subsequent tubes, more and more dye was reduced.

Although the determination of the —SH groups in heat-denatured egg albumin gave results in agreement with those of Todrick and Walker (4) by 2,6-dichlorophenol indophenol oxidation and those of Mirsky and Anson (5) by oxidation with cystine, Kuhn and Desnuelle recognize that other oxidizable groups may be present in proteins.

Greenstein (3, 6) has extended the use of porphyrindin to the determination of —SH groups liberated in proteins by the action of urea, guanidine, and their derivatives. His technique was

\* This work was aided by a grant from the John and Mary R. Markle Foundation.



somewhat different; he first treated the proteins with urea or guanidine hydrochloride at 25°, then titrated with a porphyrindin solution at this temperature. The —SH groups liberated from egg albumin in the presence of urea or of guanidine hydrochloride amounted to 1.00 and 1.24 per cent of cysteine, respectively.

The experiments reported below show that porphyrindin oxidizes not only —SH groups, but under certain conditions tyrosine also. With the technique here described a cysteine content of 1.35 per cent was found for heat-denatured egg albumin. In native egg albumin the phenolic and —SH groups are either absent or inaccessible to oxidation. Heat denaturation is associated with the liberation of —SH groups, while dispersion by guanidine hydrochloride is accompanied by the appearance of reactive —SH groups as well as reactive phenolic groups.

#### EXPERIMENTAL

Porphyrindin was synthesized as described by Kuhn and Franke (2); the condensation of HCN with acetoxime was carried out according to Porter and Hellerman (7). Recrystallization of porphyrindin<sup>1</sup> from pyridine was unsuccessful.

*Standardization of Porphyrindin Solutions*—Kuhn establishes the titer of porphyrindin solutions with *pure* cysteine in a series of Thunberg tubes at 0° and pH 7.2. This procedure gives satisfactory results. By Greenstein's method, a standard solution of cysteine hydrochloride, neutralized with ammonia to pH 7, is titrated with porphyrindin at 25°. We find that under these conditions both solutions deteriorate at 25°. However, the direct titration can be accomplished in the following manner. Porphyrindin (5 to 7 mg. per 10 cc.) is dissolved in 0.2 M phosphate buffer, pH 7.2, at 0°, and the solution filtered. 2 cc. of the porphyrindin solution are titrated, in a test-tube kept at 0°, with an aqueous solution of metal-free cysteine hydrochloride (about 50 mg. per 100 cc.) to the disappearance of the blue color.

<sup>1</sup> An absorption maximum at 653 m $\mu$  has been reported for the dye (2). Using the Pulfrich photometer, we find that Zeiss Filter S-57 gives the highest extinction coefficient (determined at room temperature with freshly prepared solutions at pH 7.2 and 9). The same result was obtained with a number of pure preparations (one kindly furnished by Dr. J. P. Greenstein).

The concentration of the cysteine hydrochloride solution is established photometrically (8) or iodometrically ((9), cf. (10)). 1 mg. of cysteine is equivalent to 1.16 mg. of porphyrindin.

Porphyrindin may also be standardized iodometrically. 2 cc. of cold porphyrindin solution, prepared as described above, are added to 3 cc. of a solution containing 0.3 gm. of KI in 0.5 N HCl, the flask being whirled during the addition.<sup>2</sup> Iodine is immediately liberated and titrated with 0.01 N thiosulfate. 1 cc. of 0.01 N thiosulfate is equivalent to 1.40 mg. of porphyrindin. A standardized porphyrindin solution may be used to establish the titer of cysteine solutions.

*Stability of Porphyrindin Solutions*—The crystalline dye is not entirely stable (1); it is best kept at low temperature. In our experience, the deterioration of porphyrindin solutions at 0° amounted to 3, 5, and 9 per cent in 1, 2, and 4 hours, respectively. At 25° deterioration occurred at the rate of about 0.5 per cent per minute. We have therefore carried out all experiments with porphyrindin at 0°, determining the titer of the porphyrindin solutions before and after each experiment.

*Reaction of Amino Acids with Porphyrindin*—Sulphydryl compounds, such as cysteine and glutathione, are stoichiometrically oxidized to the corresponding —S—S— compounds, even when an excess of porphyrindin is used. Guanidine hydrochloride has no effect on the reaction. Cysteine (0.86 mg.) was treated for 15 minutes at 0° in an evacuated<sup>3</sup> Thunberg tube, in 6.5 cc. of solution containing 2 cc. of 0.5 M phosphate buffer of pH 7.2, with 2.08 mg. of porphyrindin.<sup>4</sup> The excess porphyrindin was then titrated with a standardized solution of cysteine hydrochloride; 1.10 mg. of the porphyrindin were reduced (calculated 1.06 mg.). In a parallel experiment with the addition of 3 gm. of guanidine hydrochloride at pH 7.2 the same amount (1.10 mg.) of porphyrindin was reduced. Glutathione (2.80 mg.) was treated under the same conditions with 3.00 mg. of porphyrindin; 1.31

<sup>2</sup> Although porphyrindin is destroyed on short contact with acid, the liberation of iodine from HI is quantitative under the conditions described.

<sup>3</sup> The tubes were alternately evacuated and filled with nitrogen several times, then left evacuated up to the time of titration.

<sup>4</sup> The oxidation of cysteine is complete in 30 to 60 seconds, even if no excess of porphyrindin is added.

mg. of porphyrindin were reduced (calculated 1.28 mg.). In a parallel experiment with 3 gm. of neutral guanidine hydrochloride, 1.29 mg. of porphyrindin were reduced.

The *l* forms of cystine,<sup>5</sup> cysteic acid, tryptophane, hydroxyproline, and histidine, the *dl* forms of methionine, serine, phenylalanine, and threonine did not reduce porphyrindin when approximately 1 to 10 mg. of the amino acid and 1 to 2 mg. of porphyrindin in a total volume of about 3 cc. were allowed to stand at 0° and pH 7.2 for 15 minutes in an evacuated<sup>3</sup> Thunberg tube.

Tyrosine is oxidized by porphyrindin at 0° and pH 7.2 with the formation of a pink<sup>6</sup> oxidation product. It can be seen from Fig. 1 that the oxidation depends on the reaction time (Curve 1) and on the amount of porphyrindin present (Curve 2). The addition of 3 gm. of guanidine hydrochloride at pH 7.2 to the reaction mixture has no effect on the oxidation of tyrosine by porphyrindin (the triangles on Curve 2 represent experiments with added guanidine hydrochloride).

*Native Egg Albumin and Porphyrindin*—Native egg albumin does not decolorize porphyrindin solutions (1, 5), even in the presence of a large excess of the dye. There is no reaction when 34.3 mg. (*i.e.*, 1 micromole on the basis of a molecular weight of 34,300 (10, 11)) of native egg albumin, six times recrystallized and then dialyzed, are allowed to stand for 10 minutes at 0° and pH 7.2 in a volume of 3 cc. with 2 mg. of porphyrindin (14 microequivalents). However, if such egg albumin-porphyrindin solutions are shaken continuously for 10 minutes at 0° in evacuated<sup>3</sup> Thunberg tubes, a small amount of porphyrindin is reduced, presumably by surface-denatured egg albumin.

<sup>5</sup> Cystine and cysteic acid are extremely resistant to oxidation by porphyrindin at 0° and pH 7.2. No reduction of porphyrindin occurred when 2.5 microequivalents of these substances were treated in a volume of 2.5 cc. with approximately 25 microequivalents of porphyrindin for 2 hours. On the other hand, the sulfinic acid corresponding to cysteine and the disulfoxide of cystine are oxidized by porphyrindin at 0° and pH 7.2, although at a much slower rate than —SH compounds. Several hours were required for the reactions to be completed, even when a large excess of porphyrindin was used. A more detailed study of these reactions will be presented in a later publication, in which the possible presence of the sulfinic acid in denatured egg albumin will be considered.

<sup>6</sup> With very small amounts of porphyrindin a yellow color is obtained.

*Oxidation of Heat-Denatured Egg Albumin by Porphyrindin*—Crystalline egg albumin (34.3 mg. in 3 cc. of phosphate buffer of pH 7.2, 0.5 M) was denatured by heating in an evacuated<sup>3</sup> Thunberg tube for 10 minutes in a boiling water bath with vigorous shaking<sup>7</sup> (1). After being cooled to 0°, the tube was filled with nitrogen and a definite amount of porphyrindin was added, either as solid or dissolved in phosphate buffer. The volume was adjusted with buffer to about 5 cc.; the tube was evacuated<sup>3</sup> again and shaken for 10 minutes in ice. The excess porphyrindin was then titrated at 0° with cysteine hydrochloride solution.

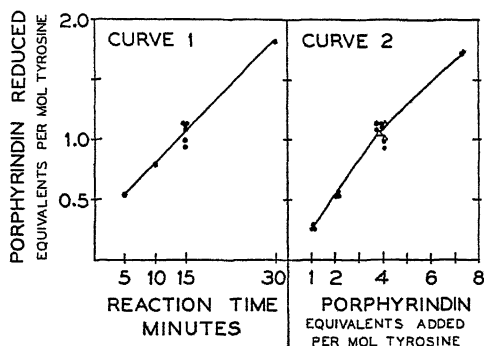


FIG. 1. Reaction of tyrosine with porphyrindin. Curve 1, reaction of 0.5 mg. of tyrosine with 1.54 mg. of porphyrindin (4 equivalents per mole), volume 6.5 cc., 0.2 M phosphate buffer, pH 7.2, 0°; Curve 2, reaction of 0.5 mg. of tyrosine with varying amounts of porphyrindin, reaction time 15 minutes, volume 6.5 cc., 0.2 M phosphate buffer, pH 7.2, 0°; the triangles represent experiments with 3 gm. of guanidine hydrochloride added.

In all the experiments with heat-denatured egg albumin (Fig. 2), even with the largest amount of porphyrindin, there was no pink or yellow color in the protein suspensions after the excess of the dye had been decolorized by cysteine. This indicates that no tyrosine was oxidized. The —SH groups determined from the amount of porphyrindin which was completely decolorized (Kuhn's "end-point") correspond to 0.43 per cent cysteine or to a transfer of 1.2 atoms of hydrogen per mole of protein. As the amount of porphyrindin in the reaction mixture is increased, more is reduced. When 10 to 27 microequivalents of porphyrindin per micromole of egg albumin are added, the apparent cysteine

<sup>7</sup> Reliable results are obtained only with fairly uniform protein suspensions.

content is approximately constant at 1.35 per cent; *i.e.*, a transfer of about 4 hydrogen atoms per mole.

When 34.3 mg. of egg albumin preparation, which had been heat-denatured, dried, and kept without special precautions (Preparation C; *cf.* (10, 12)), were suspended in 3 cc. of phosphate buffer, pH 7.2, and shaken<sup>3</sup> for 15 minutes at 0° with 39 microequivalents of porphyrindin, no porphyrindin was reduced, although the preparation yielded cysteine on HCl hydrolysis.<sup>8</sup> This negative result may perhaps be attributed to autoxidation of the denatured protein.

*Oxidation of Egg Albumin by Porphyrindin in Presence of Guanidine Hydrochloride*—Greenstein (3) treated native egg al-

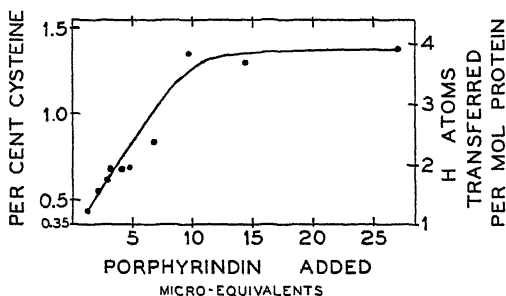


FIG. 2. Reaction of heat-denatured egg albumin with varying amounts of porphyrindin. 34.3 mg. of egg albumin (1 micromole) in 4 to 5 cc. of 0.2 M phosphate buffer, pH 7.2, 10 minutes at 0°.

bumin solutions in open tubes at 25° and pH 7 with guanidine hydrochloride for 45 minutes, then titrated with a porphyrindin solution to the appearance of the blue color. A maximum of titratable groups, corresponding to 1.28 per cent cysteine, was reached after treatment with 0.8 gm. of guanidine hydrochloride per 2 cc. of protein solution.

All of our experiments were carried out in Thunberg tubes in an inert atmosphere.<sup>3</sup> In the first series of experiments (Fig. 3, experiments represented by triangles) 34.3 mg. of egg albumin in 1 cc. of phosphate buffer of pH 7.2 were treated with 3 gm. of guanidine hydrochloride for 1 hour at 25°. The tubes were

<sup>8</sup> Hydrolysis of this egg albumin preparation with 6 N HCl yielded 0.25 per cent cysteine (12). A new method of hydrolysis, with HCl containing large amounts of urea, yielded 0.5 per cent cysteine (unpublished experiments of Brand and Kassell).

cooled to 0°, varying amounts of porphyrindin in 1 to 2 cc. of solution were added, and after 10 minutes at 0° the excess porphyrindin was titrated with cysteine hydrochloride. In the experiments represented by dots in Fig. 3, the same amount of egg albumin and guanidine hydrochloride in 2 to 3 cc. of buffer was treated at once with graded amounts of porphyrindin at 0° for 15 minutes; then the excess porphyrindin was titrated with cysteine hydrochloride.

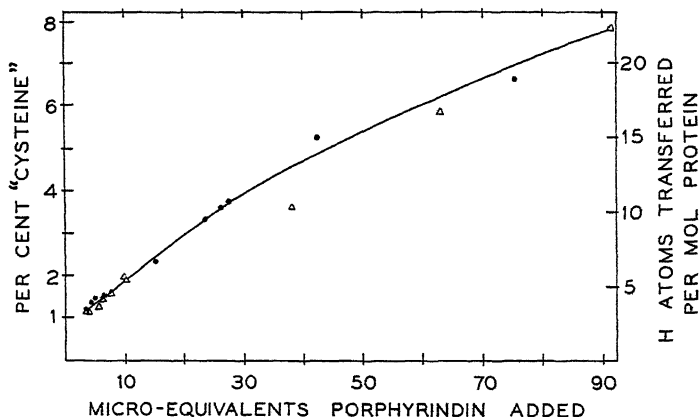


FIG. 3. Reaction of egg albumin with porphyrindin in the presence of guanidine hydrochloride. Dots, reaction of 34.3 mg. of native egg albumin (1 micromole) plus 3 gm. of (neutralized) guanidine hydrochloride with varying amounts of porphyrindin, volume 4 to 5 cc., 0.2 M phosphate buffer, pH 7.2, 15 minutes at 0°; triangles, 34.3 mg. of native egg albumin in 1 cc. of 0.5 M phosphate buffer, pH 7.2, first treated for 1 hour at 25° in an inert atmosphere with 3 gm. of (neutralized) guanidine hydrochloride. After cooling to 0° varying amounts of porphyrindin in 1 to 2 cc. of diluted buffer were added and allowed to react 10 minutes at 0°.

In both experiments the shape of the curve indicates that the reaction was not complete, even when the amount of porphyrindin reduced corresponded to a "cysteine" content of about 8 per cent, or to a transfer of 22 atoms of hydrogen per mole of egg albumin. However, when titration was completed, all the tubes which had been treated with more than 10 microequivalents of porphyrindin showed the pink color<sup>9</sup> characteristic of the oxidation product of tyrosine.

<sup>9</sup> Balls and Lineweaver (13) noted the production of a red color when a large quantity of porphyrindin was added to papain.

The heat-denatured, oxidized egg albumin (Preparation C), which did not react with porphyrindin (*cf.* above), was shaken at 0° and pH 7.2 for 15 minutes with 3 gm. of guanidine hydrochloride and varying amounts of porphyrindin. Under these conditions 1 micromole of the egg albumin reacted with porphyrindin equivalent to the transfer of 5 and 7 hydrogen atoms per mole when 16 and 30 microequivalents of porphyrindin, respectively, were added. The protein did not seem to dissolve, and after titration of the excess porphyrindin, the protein particles were distinctly yellow.<sup>6</sup> It therefore seems likely that part of the porphyrindin was reduced by tyrosine.

#### SUMMARY

1. Porphyrindin solutions are relatively unstable above 0°.
2. The oxidation of cysteine by porphyrindin does not go beyond the —S—S— stage at 0° and pH 7.2.
3. Tyrosine is oxidized by porphyrindin at 0° and pH 7.2 with the formation of a pink oxidation product.
4. Native egg albumin is stable towards porphyrindin. In heat-denatured egg albumin, —SH groups are oxidized by porphyrindin. In egg albumin dispersed by guanidine hydrochloride, —SH groups as well as phenolic groups are oxidized by porphyrindin.

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## HEPARINASE

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Howell's first physiological experiments with heparin indicated that its anticoagulant effect following intravenous injection wears off rapidly. The results of a quantitative study of this phenomenon by the author (1) emphasized the rapidity of this process. Howell and MacDonald (2) have suggested that the injected heparin is excreted. Other possibilities are that the heparin is stored in some tissue, probably combined with protein, or that it is neutralized by the secretion of coagulant substances. A fourth possibility is destruction of the anticoagulant by enzyme action. The failure to find any evidence by direct experiment (1) for the operation, to any great extent, of the first three mechanisms in the dog led me to search in mammalian tissues for an enzyme which would inactivate heparin and hence might be responsible for the process. This is the subject of the present communication. Such an enzyme is readily obtained from rabbit liver by glycerol extraction, followed by fractionation of the extract with ammonium sulfate. Since the first substrate used is heparin, the name "heparinase" has been adopted.

Tissue extracts may inactivate heparin owing to the presence of (1) coagulant substances, (2) protein, or (3) heparinase. The coagulant substance or substances present have a direct effect on the clotting system used for assaying the heparin and can be distinguished in this way. Their activity is destroyed by heat. While thrombokinase is the usual coagulant present, other substances with a kinase action, or thrombin, may be present.

The phenomenon of the inactivation of heparin by proteins is one aspect of the reaction of heparin with proteins discovered and extensively investigated by Fischer (3). This discovery has been



of the utmost importance for an appreciation of the properties of heparin. Inactivation of the heparin occurs if the affinity of the protein for heparin is great enough for it to compete with the clotting system for the anticoagulant. From the work of Fischer, inactivation by protein can be distinguished from the other types by the effect of heat, the effect of pH, and the reversible nature of the inactivation. Heating the extract increases the inactivation of the heparin; *i.e.*, after denaturation the protein is able to bind more heparin. Protein inactivation takes place anywhere on the acid side of the isoelectric point of the protein (no pH optimum). It can be reversed by suitable treatment with alkali.

Owing to these complicating factors, it is difficult to demonstrate the presence of heparinase in simple extracts. Further, special control experiments are required with all extracts to insure that only the true enzymic inactivation is involved. It must also be remembered that heparin is easily inactivated by heating in acid solution (4). For this reason suitable controls should always be set up and considerable care exercised in the use of heat to destroy the enzyme when samples are removed from the reaction mixture. Enzymic inactivation of heparin can be distinguished from the other two types of inactivation, since the activity of the enzyme is destroyed by heat; yet the enzyme has no direct effect on the clotting system. However, a more practical way to distinguish the enzyme inactivation is that it is progressive, whereas, after a brief initial period, further incubation of heparin with protein or kinase does not increase the amount of heparin inactivated. Also, it alone has a definite and narrow pH optimum (5.3 to 6.8). By use of these last two criteria, it is possible to demonstrate the action of heparinase even in the presence of the other two types of inactivating substances.

#### EXPERIMENTAL

*Preparation of Enzyme*—While various sources and types of extracts were tried, only the final method of preparation will be described. The livers (3 kilos) are removed from a number of rabbits, finely minced, 2 volumes of glycerol added, and the mixture stirred vigorously for 2 hours. It is then stored in the ice box. As required, 1 liter of this mixture is removed and dialyzed overnight against running water. It is then dialyzed for several

hours against distilled water. The cell pulp is filtered off with suction, with celite No. 535 (Johns-Manville) as a filter aid. The muddy filtrate is clarified by filtering with Johns-Manville standard filter-cel. A clear reddish filtrate is obtained. An equal volume of saturated ammonium sulfate is added and the mixture filtered on a soft paper. The precipitate containing the heparinase is transferred to a dialyzing bag and dialyzed as before. Sufficient sodium chloride and tricresol are added to the suspension to give a concentration of 1.0 and 0.3 per cent respectively and undissolved protein is removed by centrifuging. The resulting heparinase preparation is stored in the ice box. It gradually loses activity over a period of several weeks. 1 cc. of this preparation causes destruction of 50 units, *i.e.* 0.5 mg., of standard heparin in 24 hours.

*Assay of Enzyme*—In order to determine the potency of the extract, 1 cc. of the extract plus 8 cc. of physiological saline containing tricresol is taken and the pH of the mixture adjusted to 6.0 with 0.1 N HCl. 1 cc. (100 units) of a standard heparin solution in saline is added. Two similar tubes are set up as controls, in one of which the heparin is omitted, in the other the enzyme. The tubes are incubated at 37° for 24 hours. The heparin content of the solutions is then assayed. The two control tubes are mixed just before assaying. Considerable time (about an hour) elapses between such mixing and the addition of the blood to the assay tubes, so that this control will compensate for any anti-heparin action due to kinase or protein in the enzyme solution. When the boiled extract has been found to have no effect on heparin (*i.e.*, absence of protein inactivation), it is possible to set up a single control tube containing boiled enzyme plus heparin. Controls of this nature have not been used to any great extent in the work reported, since few extracts do not show considerable inactivation of heparin by denatured protein although there is no inactivation by protein in the unheated extract. All data reported represent the difference in inactivation between the experimental and control values. Inactivation of the controls in Figs. 1 and 2 was relatively slight. While the 24 hour value must be considered a qualitative rather than a quantitative test for the enzyme, it suffices for preliminary studies. More accurate measurements of enzyme activity based on the measurement of reaction velocities

rather than equilibrium values are not possible at present, owing to the technical difficulties involved in biological assays of the required degree of accuracy.

*Heparin Assay*—The heparin content of the enzyme digest was assayed by the Charles and Scott (4) modification of the Howell method. For some of the experiments, oxalated beef blood clotted with thrombin was used in place of the cat blood of the Howell method. This has been found to be more satisfactory for experiments of this nature, as the end-point is much sharper. The heparin potency is found by comparison with that of a standard solution. A series of dilutions of the standard is set up with each unknown solution. For the cat assay, the end-point is usually about 0.25 to 0.75 unit. The digest is diluted to a concentration of 5 units per cc. on the basis of the original heparin concentration. 0.08, 0.10, 0.12, 0.15, 0.20, and 0.30 cc. of the digest are then taken in the assay tubes and the volume made up to 0.30 cc. with saline. With a 0.5 unit end-point, the end-point found with these successive tubes then represents 125, 100, 83, 67, 50, and 33 per cent of the original heparin activity. With the control solution, as it is not necessary to allow for such a drop in activity, the same amounts of a dilution equivalent to 3.3 units per cc. may be taken. For the thrombin assay, the end-point is established at 0.10 unit. Hence the 5 unit per cc. solution is diluted 1:5, and the same volumes as above taken. As the end-point with the thrombin assay is much sharper, more tubes to include volumes intermediate between these are taken. Direct measurements showed that even the most acid digests had no effect on the pH of the blood in the dilutions used for assay. pH measurements were made with the Beckman glass electrode. All solutions and the saline used contained 0.3 per cent tricresol as a preservative. The heparin used was beef heparin in the form of the sodium salt, prepared from the crystalline barium salt and supplied by the Connaught Laboratories. The unit is that used by them and has been found to be equal to the potency of 0.01 mg. of the crystalline barium salt of Charles and Scott (5).

*Progress and pH-Activity Curves of Enzyme*—From the previous discussion it is clear that in practice the essential data necessary to distinguish inactivation of heparin by heparinase from the more general types of inactivation by tissue extracts are the prog-

ress and pH-activity curves; *i.e.*, the demonstration of an increase in inactivation with time and also a true pH optimum for the reaction. Progress curves observed with the enzyme are shown in Fig. 1. Controls were set up as described above and a sample from the controls was also assayed. Inactivation in the control tubes was found to be negligible. As can be seen, with increasing times of incubation the amount of heparin inactivated increases, the curve being of the usual enzyme type. Further, as would be expected, when the amount of enzyme is increased, the rate of inactivation of the heparin is correspondingly increased, as judged by the values observed after 2 hours and 24 hours incubation. After 24 hours at 37°, the enzyme appears to become largely inactivated, as very little further action takes place. As mentioned in the method of preparation, the enzyme is not very stable in aqueous solution, and gradually loses its activity even when stored at low temperatures. Since the enzyme is largely inactivated after 24 hours under the conditions of the experiment, the equilibrium value obtained is close to the 24 hour value and hence depends on the amount of enzyme used, as shown in Fig. 1. In order to obtain true equilibrium values, concentrated preparations of the enzyme and optimum conditions for its activity must be used, in order that there be complete inactivation of the heparin before much inactivation of the enzyme has occurred. The pH of the enzyme mixture in this experiment was 6.8 and outside the optimum range for the enzyme. The pH-activity curve is shown in Fig. 2. Digests and controls were made up in the usual manner and the heparin remaining after 48 hours incubation determined. The pH was adjusted by the addition of 0.1 N HCl. Measurements of the pH of the mixture at the time of assay showed that there had been no change in pH; *i.e.*, the buffering power of the enzyme extract was great enough to keep the pH constant. To one series of tubes, 1 cc. of phosphate-acetate buffer was added. This appeared to have little effect on the activity of the enzyme. Inactivation of heparin in the controls was observed in the digests at pH 2.6 and 3.5 but it was relatively slight. It can be seen that inactivation of heparin by the enzyme occurs only in the pH range 3 to 7. On either side of this the enzyme is inactive. The optimum pH range for the enzyme appears to be from 5.3 to 6.8. Its activity decreases very rapidly

on the alkaline side of the optimum, no activity being observed at pH 7.3. In the early investigations a number of extracts were tested with negative results. It is clear that this was partly due to the tests being at pH values outside the narrow optimum range. The rapid decrease in the activity of the enzyme on the alkaline side of the pH optimum is particularly striking. The decrease on the acid side is more gradual.

*Nature of Reaction*—From our present knowledge of the chemistry of heparin, it is conceivable that heparinase might inactivate heparin either by attacking the sulfate groups or the amino group

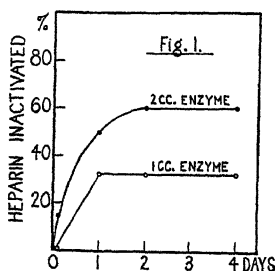


FIG. 1. Rate of inactivation of heparin by heparinase. Heparin 100 units in a 10 cc. digest mixture. pH 6.80.

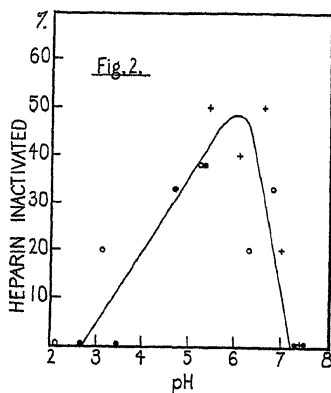


FIG. 2. Effect of pH on the inactivation of heparin by heparinase. 1 cc. of enzyme + 100 units of heparin. Total volume, 10 cc. pH established by addition of 0.1 N hydrochloric acid. The results of three separate experiments are shown. For the points indicated by crosses, 1 cc. of phosphate-acetate buffer was included in the reaction mixture.

or a linkage in the carbohydrate portion of the molecule. As shown by Chargaff (6), heparin combines with protein through its sulfate groups. As its anticoagulant action is dependent on the formation of a heparin-protein compound (Quick (7)), it appears not unlikely that *in vivo* the sulfate groups of heparin are bound and thus not available for the action of a sulfatase. To test the possibility of a sulfatase, however, 10 cc. digests were taken, the protein precipitated with picric acid, and the supernatant tested with barium chloride. No trace of precipitate or

turbidity was observed, whereas apparently complete precipitation of the barium sulfate was observed in control digests to which 1 mg. of sodium sulfate (equivalent to the sulfate in the heparin inactivated) had been added. Hence no inorganic sulfate is formed during the inactivation. Due to the high reducing value of the enzyme preparation, it is not possible at present to determine any change in reduction. It is not improbable, however, that the action of the enzyme is directed at the carbohydrate portion of heparin.

#### SUMMARY

The inactivation of heparin by tissue extracts is discussed. The preparation and properties of an enzyme, heparinase, which inactivates heparin are described. The enzyme is prepared from rabbit liver. It is precipitated by half saturation with ammonium sulfate, and its pH optimum is in the range 5.3 to 6.8.

The author is greatly indebted to Mr. J. Wilson of the Connaught Laboratories for many of the biological assays.

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## THE EFFECT OF INSULIN ON MUSCLE RESPIRATION\*

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According to Krebs and Eggleston (1) insulin accelerates the respiration of muscle tissue *in vitro*. The effect was observed with pigeon breast muscle suspended in 0.1 M phosphate buffer, pH 6.8, supplemented with muscle juice, and citric or some dicarboxylic acid. In this system insulin was the limiting factor. Shorr and Barker (2) studied the effect on rabbit, cat, and dog muscle, and failed to observe any response to insulin. In pigeon muscle they observed a 20 per cent increase, in contrast to the 90 per cent increase reported by Krebs and Eggleston. Banga and co-workers (3) observed stimulation due to insulin in certain brain preparations fortified with fumarate, adenylic acid, and pyruvate.

Like Shorr and Barker we have failed to observe a marked increase in respiration under the experimental conditions originally outlined by Krebs and Eggleston, but when a Ringer-phosphate medium was used, insulin increased respiration, particularly in the breast muscles of depancreatized pigeons (4). A detailed study of this insulin effect has therefore been made, including the character of the respiration, the response to various di- and tri-carboxylic acids, the effect of pancreatectomy, and a correlation of insulin effects *in vivo* and *in vitro*.

### Methods

The oxygen consumption of minced pigeon breast muscle was measured in the usual way in a standard Warburg respirometer (4, 5). The medium was a Ringer-phosphate buffer free of calcium (6), pH 7.4, containing 0.2 per cent glucose. Approximately

\* Supported by the Jonathan Bowman Cancer Fund and the Wisconsin Alumni Research Foundation.



150 mg. of fresh tissue were suspended in 2 cc. of medium. The medium was supplemented with 0.1 or 0.2 cc. of muscle juice (*Kochsaft*) (6), and a di- or tricarboxylic acid. The following acids were studied: fumaric, malic, succinic,  $\alpha$ -ketoglutaric, citric, and glutamic. The solutions were brought to pH 7.4 before addition to the medium, and the final concentration of the "acid" was 0.001 or 0.005 M. Both the muscle juice and the solutions of dicarboxylic acid were prepared at weekly intervals.  $\alpha$ -Ketoglutaric acid was always dissolved immediately prior to use.

Two forms of insulin were available for these studies, amorphous insulin,<sup>1</sup> and a commercial preparation, Iletin, containing 80 units per cc. In the earlier experiments up to 40 units of insulin were used per 2 cc. of medium, but these higher amounts frequently inhibited the respiration of normal muscle (4). The optimum concentration appeared to be 4 units per 2 cc. of medium, and this amount was later adopted as standard. The experiments were continued for 4 hours. This long experimental period raised the question whether bacterial contamination affected respiration results. Accordingly, bacterial plate counts were made every hour for 9 hours. Under the usual laboratory conditions, with clean glassware and instruments, the number of organisms present was too small to affect respiration until after the 5th hour.

To obtain muscle low in insulin, birds were depancreatized 1½ to 4 weeks prior to use. Complete surgical removal of the pancreas was attempted, but was probably never achieved, as small bits of pancreas containing islet tissue were observed on autopsy. However, at least 90 to 95 per cent of the pancreas had been removed.

*Effect of Insulin on Respiring Muscle*—Insulin increased the respiration of pigeon breast muscle both in the presence and absence of supplements which themselves also increased respiration appreciably.

Either insulin, fumarate, or muscle juice alone increased the oxygen uptake about 20 per cent over that of the control. Insulin and fumarate or insulin and muscle juice were somewhat more effective than either alone, and the three together were more

<sup>1</sup> We are indebted to Dr. G. H. A. Clowes of Eli Lilly and Company for this preparation.

effective than any two, causing an increase of 80 per cent over the unsupplemented control. The effect of insulin was most pronounced during the latter part of the experiment. During the 3rd and 4th hours respiration had practically ceased in the unsupplemented control tissues, whereas in media containing insulin, either in the presence or absence of other supplements, muscle continued to respire appreciably. Insulin, therefore, tended to preserve respiration rather than to increase it. A typical experiment with the various controls is given in Table I.

TABLE I\*

*Effect of Insulin and Other Supplements on Oxygen Consumption of Breast Muscle from Depancreatized Pigeon*

O<sub>2</sub> = total c.mm. of oxygen consumed per mg. of dry weight of tissue during the time *t* (6).

Supplements	O <sub>2</sub> (4 hrs.)	QO <sub>2</sub> (4th hr.)
None, control .....	20.6	0.9
Insulin (1 unit) .....	28.0	3.0
Fumarate (0.001 M) .....	26.1	1.9
Muscle juice (0.2 cc.) .....	25.4	2.2
Malonate (0.001 M) .....	4.7	0.3
Insulin + fumarate .....	32.8	4.5
“ + muscle juice .....	32.0	4.4
“ + malonate .....	5.1	0.3
Fumarate + muscle juice .....	28.0	2.5
“ + malonate .....	25.7	1.8
Muscle juice + malonate .....	15.5	1.3
Insulin + fumarate + muscle juice .....	37.2	6.4
“ + “ + “ + “ + malonate .....	36.9	6.3

\* To conserve space the results have been skeletonized. Detailed protocols of preliminary experiments are given elsewhere (4).

*Importance of Di(Tri)-Carboxylic Acids*—Apparently active di(tri)-carboxylic acids must be present in the respiring mixture for insulin to exert its effect. In addition to fumaric acid, the following were used to “sensitize” muscle to insulin: succinic, malic, citric (Fig. 1),  $\alpha$ -ketoglutaric, and glutamic (Table II) acids. Since these substances are normally present in muscle, it is possible to observe an increase in respiration when insulin alone is added, though the effect is enhanced by the presence of

more dicarboxylic acid. When the dicarboxylic acid is effectively inactivated by malonate, respiration is strongly inhibited both in the presence and absence of insulin (Table I). The addition of fumarate restores respiration and sensitivity to insulin, even in the presence of malonate, since fumarate nullifies the action of malonate in equivalent concentrations (5).

The degree of malonate inhibition in systems containing insulin and other di(tri)-carboxylic acids varied with the kind and amount

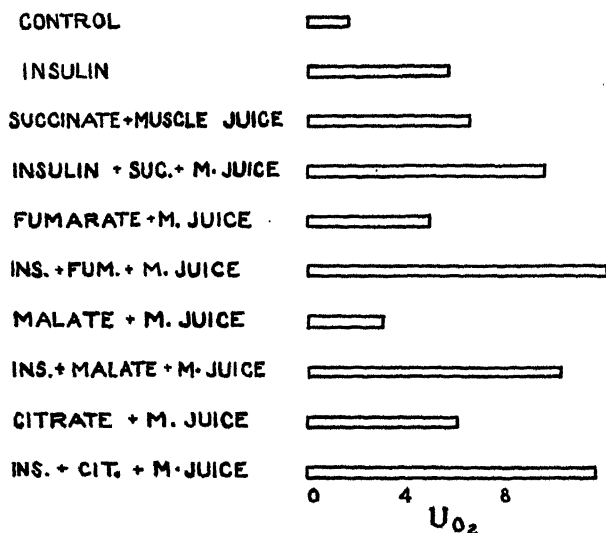


FIG. 1. The effect of insulin and supplements on the oxygen consumption of breast muscle from a depancreatized pigeon, during the 3rd and 4th hours of the experiment. 0.001 M succinate, fumarate, malate, and citrate were used, 0.2 cc. of muscle juice, and 1 unit of amorphous insulin. Blood sugar before pancreatectomy was 197 mg. per cent, after 11 days 282 mg. per cent.

of acid present, in accordance with the ability of the acid to compensate for the malonate effect in the absence of insulin (5). In systems fortified with fumarate, muscle juice, and insulin, no inhibition within experimental error was observed in the presence of equimolar amounts of malonate (Tables I and III). When citrate was substituted for fumarate, however, respiration was inhibited 68 per cent. In the presence of insulin only, malonate

inhibition was 88 per cent (Table III). Glutamic acid, like citric, failed to compensate for malonate (5), and insulin systems containing glutamic acid were also markedly sensitive to malonate (Table II). Malic and  $\alpha$ -ketoglutaric acids were like fumaric acid in protecting insulin respiration from malonate, whereas succinic acid was somewhat less effective.

Another factor in respiration appeared to be cocarboxylase, since it increased respiration in the presence of other supplements (Table II). Cocarboxylase<sup>2</sup> alone had no effect on the respira-

TABLE II

*Effect of Insulin, Glutamic and  $\alpha$ -Ketoglutaric Acids, and Other Supplements on Respiration of Pigeon Breast Muscle*

Supplements	O <sub>2</sub> (4 hrs.)	QO <sub>2</sub> (4th hr.)
	<i>c.mm.</i>	
Control.....	15.4	1.2
Glutamic acid (0.005 M).....	24.2	2.7
Muscle juice (0.2 cc.).....	17.7	1.5
Insulin (4 units).....	14.5	0.5
Cocarboxylase (100 $\gamma$ ).....	13.4	0.4
Malonate (0.005 M).....	4.6	0.1
Glutamic acid + muscle juice.....	38.5	5.0
“ “ + “ “ + insulin.....	44.2	9.6
“ “ + “ “ + “ + malonate..	12.7	0.8
“ “ + “ “ + “ + cocar-		
boxylase.....	50.0	11.0
$\alpha$ -Ketoglutaric acid (0.005 M).....	18.4	2.4
“ “ + muscle juice.....	24.6	2.8
“ “ + “ “ + insulin.....	40.0	8.0

tion of normal muscle, although it did increase the respiration of muscle from a depancreatized pigeon (Table III).

*Effect of Pancreatectomy*—A marked difference between the muscles of normal and depancreatized birds was observed, both in the oxygen uptake and in the sensitivity to insulin. To a considerable extent it was possible to correlate these differences with the time elapsed from pancreatectomy until the bird was used for the experiment (Table IV). The birds were grouped into five groups of two to five each. Group 1 consisted of five representa-

<sup>2</sup> We are indebted to Professor C. A. Elvehjem for this preparation.

tive normal birds; Group 2, three pigeons used in the 2nd week after pancreatectomy; Group 3, three pigeons in good condition, used during the 3rd week after pancreatectomy; Group 4, two extremely emaciated birds used during the 3rd week after pancreatectomy; and Group 5, three healthy birds used during the 4th week after pancreatectomy.

TABLE III

*Effect of Malonate on Respiration of Pigeon Breast Muscle Fortified with Insulin, Fumarate, and Citrate*

Pigeon No.	Supplements	O <sub>2</sub> (4 hrs.)	Per cent inhibition by mal- onate
		<i>c.mm.</i>	
4. Depan- creatized	None, control	11.1	
	Malonate (0.001 M)	5.2	53
	Insulin (4 units)	20.0	
	“ + malonate	5.9	71
	Fumarate (0.001 M)	18.7	
	“ + malonate	18.1	3
	“ + muscle juice	21.1	
	Insulin + “ “ + fumarate	30.1	
7. Depan- creatized	“ + “ “ + “ + mal- onate	29.0	4
	None, control	10.6	
	Malonate (0.005 M)	3.0	72
	Insulin (4 units)	18.3	
	“ + malonate	2.2	88
	Citrate (0.005 M)	15.5	
	“ + malonate	5.5	64
	“ + muscle juice	17.9	
	Insulin + citrate + muscle juice	24.5	
	“ + “ + “ “ + malonate	7.7	68
	Coccarboxylase (100 γ)	19.3	
	Citrate + insulin + muscle juice + cocar- boxylase	31.0	

During the 2nd week of recovery, control respiration was actually somewhat higher than that of muscle from normal pigeons and the sensitivity to insulin had increased markedly. Insulin increased respiration 62 per cent in the unsupplemented tissues and 40 per cent in the supplemented tissues (Group 2), as com-

pared to increases averaging 20 and 16 per cent respectively in normal muscle (Group 1). Although the sensitivity to insulin had increased, the per cent of increase due to the complete system

TABLE IV

*Effect of Pancreatectomy on Oxygen Uptake of Pigeon Breast Muscle and Its Sensitivity to Insulin*

Group No.	Pigeon No.		Blood sugar	O <sub>2</sub> uptake of control (un-supplemented tissue)		Per cent change in 4 hr. O <sub>2</sub> uptake upon addition of various supplements		
				1st hr.	4th hr.	Insulin to control	Insulin + C <sub>6</sub> -C <sub>6</sub> acid + muscle juice to control	Insulin to C <sub>6</sub> -C <sub>6</sub> acid + muscle juice
		days	mg. per cent	c.mm.	c.mm.			
1. Normals	7		190	9.9	18.2	+36	+94	0
	8		180	12.6	26.2	+51	+88	+30
	9		187	8.7	12.2	+17	+195	+12
	11		184	9.1	17.1	+10	+182	+16
	13		210	12.1	26.6	-5	+18	+19
Average.....			190	10.5	20.1	+20	+116	+16
2. Depancreatized	1	9	210	12.2	19.1	+100	+100	+16
	2	11	216	14.7	28.1	+47	+110	+69
	3	11	282	12.5	20.6	+40	+85	+32
Average.....			236	13.1	22.6	+62	+98	+40
3. Depancreatized	4	17		7.4	11.1	+80	+171	+43
	6	17	290	5.4	7.9	+14	+118	+14
	7	19	170	7.8	10.6	+73	+131	+161
Average.....			230	6.9	9.9	+56	+140	+73
4. Depancreatized	10	15	205	6.1	7.7	-18	+137	+57
	5	20	220	5.3	6.0	-10	+87	+44
Average.....			213	5.7	6.9	-14	+112	+51
5. Depancreatized	9	21	240	10.7	19.8	-24	+13	-28
	8	24	238	9.6	19.5	-15	+52	-14
	11	26	243	10.8	20.2	-22	+25	-18
Average.....			240	10.4	19.8	-20	+30	-20

—di(tri)-carboxylic acid, muscle juice, insulin—had decreased from an average of 116 to 98 per cent. This meant that the sensitivity of the muscle to supplements other than insulin had decreased appreciably, presumably because insulin was a limiting factor in this tissue. During the 3rd week following pancreatectomy, control respiration decreased markedly; the oxygen uptake for the 1st hour averaged 6.9 c.mm. as compared to 10.5 for normal muscle, but the sensitivity to insulin persisted. Insulin alone increased respiration 56 per cent; insulin in the presence of a di(tri)-carboxylic acid and muscle juice increased respiration 73 per cent. The complete system increased respiration 140 per cent. Thus when suitable supplements were added, respiration was nearly equal to that of normal tissues similarly supplemented, indicating that the respiratory capabilities of the muscle had been unimpaired. Presumably the lower respiration of the unsupplemented tissue was due to a lack of other essential factors as well as insulin.

Somewhat different results were obtained with the muscles of two extremely emaciated birds (Group 4), likewise used during the 3rd week following pancreatectomy. The oxygen uptake was approximately half that of normal muscle, averaging only 5.7 c.mm. The unsupplemented tissue did not respond to insulin, an average inhibition of 14 per cent being observed. However, in tissue supplemented with dicarboxylic acid and muscle juice, the addition of insulin produced an increase of 51 per cent, and the fully supplemented tissue gave an increase over the unsupplemented tissue averaging 112 per cent. It thus appeared that the muscle from the emaciated birds had been so deficient in other factors that insulin activity could not be demonstrated until they were supplied.

Pigeons which survived 4 weeks following pancreatectomy were lively and appeared to be fully recovered. The oxygen uptake of the muscle tissue also was similar to that of normal birds, but the response to insulin and the dicarboxylic acids was markedly less (Table IV, Group 5). Insulin alone inhibited respiration 20 per cent when added either to the unsupplemented tissues or to tissues supplemented with acid and muscle juice. The respiration of tissue fully supplemented with insulin, dicarboxylic acid, and muscle juice was only increased 30 per cent above that of the

control, an increase far lower than is usually observed. Thus it appeared that whereas the tissues from these birds had a normal oxygen uptake, the dicarboxylic acids exerted very little effect, and insulin actually inhibited respiration. Since the tissue seemed to be respiring at a greater rate during the 4th week than the 3rd, it is possible that the restored respiration involved some other mechanism than that concerned with insulin and the dicarboxylic acids.

*Action of Treated Insulin*—The question arose whether the effectiveness of insulin preparations in muscle respiration could be correlated with their action in lowering blood sugar in rabbits. Accordingly, insulin samples were treated to destroy their effectiveness in the living animal, and their activity on muscle was tested as before. The treatments included boiling for 30 minutes at pH 7.0, and incubation for 3 hours at 40° in the presence of M/30 alkali. When levels equivalent to 4 or 8 units of insulin were given, the treated samples failed to lower the blood sugar of rabbits, whereas 4 units of the untreated insulin lowered blood sugar markedly and produced convulsions. The treated insulin preparations, neutralized before use, were inactive in stimulating muscle respiration (Table V). Similar results were obtained with normal muscle and muscle from depancreatized birds.

*Respiratory Quotients*—Since the respiration experiments extended over a 4 hour period, it was desirable to know whether the oxygen uptake represented a true respiration, particularly with the highly fortified tissues. The respiratory quotient was therefore determined during various intervals of the experimental period on muscle from both normal and depancreatized pigeons. Muscle from normal pigeons had a normal R.Q., varying from 0.89 to 0.98 during the 1st hour of the experiment, regardless of whether the tissue was unsupplemented, or supplemented with insulin, fumarate, and muscle juice (Table VI). During the 3rd and 4th hours the R.Q. of the unsupplemented control decreased to practically zero, indicating cessation of true respiration, whereas the R.Q. of tissue supplemented with insulin, fumarate, and muscle juice remained normal at 1.05. With muscle from depancreatized pigeons, the respiratory quotients were quite comparable. During the 1st hour of the experiment the R.Q. of unsupplemented or supplemented tissues remained between



0.89 and 1.1. In the 2nd hour of the experiment there appeared to be no significant change, but in the 3rd hour of the experiment, the R.Q. of the unsupplemented control dropped to zero, whereas in the presence of insulin, or insulin plus acid plus muscle juice, no such decrease was observed. In other words, a prolonged uptake of oxygen in the presence of insulin, etc., indicated a prolongation of true respiration.

*Insulin versus Malonate in Vivo*—The inhibition of insulin action by malonate *in vitro* (Table III) raised the question whether malonate might not also hinder the action of insulin in the living

TABLE V  
*Effect of Treated Insulin on Respiration of Pigeon Breast Muscle*

	O <sub>2</sub> (4 hrs.)	Extra O <sub>2</sub> due to insulin
	c.mm.	c.mm.
Normal pigeon		
Fumarate (0.005 M) + muscle juice + untreated insulin (4 units).....	35.5	+6.1
Fumarate (0.005 M) + muscle juice + insulin (boiled) (0.005 " ) + " " + " (alkali-treated).....	29.3	-0.1
Fumarate + muscle juice (no insulin).....	29.8	+0.4
Depancreatized pigeon	29.4	
Fumarate (0.005 M) + muscle juice + untreated insulin (4 units).....	18.2	+6.6
Fumarate (0.005 M) + muscle juice + insulin (boiled) (0.005 " ) + " " + " (alkali-treated).....	12.8	+1.2
Fumarate (0.005 M) + muscle juice (no insulin).....	10.2	-1.4
	11.6	

animal. Accordingly, a 15 per cent solution of neutralized sodium malonate was injected subcutaneously into rabbits, either before or simultaneously with a solution of insulin. The sugar content of the blood was then determined at intervals, by the Shaffer-Hartmann method. Both fed and fasted rabbits were used. The exact dosages and the results of the experiments are presented in Table VII. The blood sugar of fed rabbits which received 4 units of insulin fell rapidly until the animals went into convulsions (Rabbits 1, 2, and 3). Fed Rabbits 4, 5, and 6 received the same amount of insulin, but in 2 cc. of 1 M malonate solution. Their

blood sugar showed some decrease, but much less than when insulin was given alone. Furthermore, none of these rabbits went into convulsions. Similar results were obtained with fed Rabbit 7, which received malonate 15 minutes before the insulin. Fed Rabbits 8 and 9 received only malonate and their blood sugar increased somewhat.

TABLE VI  
*Respiratory Quotient of Pigeon Breast Muscle*

	Pigeon No.	Supplement*	1st hr.	2nd hr.	3rd hr.
Normal	1	None, control	0.91		
	2	Insulin + fumarate + muscle juice	0.95		
	3	None, control	0.89		
	14	" "	0.92		0.15
	14	Insulin + fumarate + muscle juice	0.98		1.05
Depancrea- tized	4	None, control	0.91		0.0
	4	Insulin	0.89		0.92
	4	" + fumarate + muscle juice	0.97		1.90
	5	None, control	1.02		
	5	Insulin	0.96		
	5	" + $\alpha$ -ketoglutaric acid + muscle juice	0.96		
	6	None, control	0.89	0.88	0.0
	6	Insulin	0.94		1.30
	6	" + citrate + muscle juice	0.89	0.92	1.02
	7	None, control	1.00	1.03	0.0
	7	Malonate + citrate	1.10		
	7	Insulin + citrate + muscle juice	1.09		1.3

\* Insulin used = 4 units; muscle juice used = 0.2 cc.; di(tri)-carboxylic acids = 0.005 M.

Similar results were obtained with rabbits fasted for 24 hours. Malonate retarded the action of insulin in lowering blood sugar for about 1 hour (Rabbits 6 through 9, as compared with Rabbits 1 to 5), but as time went on the malonate effect wore off, and the blood sugar decreased. With the rabbits receiving malonate without added insulin (Rabbits 10 through 14) there was a tendency for the blood sugar to rise slightly. Rabbits 12 and 13,

TABLE VII

*Effect of Insulin and Malonate on Blood Sugar of Rabbit*

Rabbit No.	Treatment	Blood Sugar			
		0 hr.	1 hr.	2 hrs.	4 hrs.
Fed rabbits (2-2.5 kilos)					
		mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	4 units insulin	104			39*
2	Same	101	80	56*	
3	"	120	92	61	40*
4	4 units insulin in 2 cc. 1 M malonate	116			113
5	Same	142			111
6	"	96	75	75	
7	10 cc. 1 M malonate, 15 min. later 4 units insulin	103	85	90	
8	10 cc. 1 M malonate at 0 hr., 10 cc. at 1 hr.	115	143	150	155
9	10 cc. 1 M malonate at 0 hr., 5 cc. at 1 hr.	117	128	148	139
Fasted (24 hrs.) rabbits 2-2.5 kilos in weight					
1	4 units insulin	86	26*		
2	Same	93	46	47	54*
3	"	82	58	39*	
4	"	98	52	45	39*
5	"	83	51	42	35*
6	10 cc. 1 M malonate, 15 min. later 4 units insulin	96	83	55	37*
7	Same	100	104	61	64
8	20 cc. 1 M malonate, 15 min. later 4 units insulin	83	70	66	36*
9	Same	77	85	Died, no convulsions	
10	10 cc. 1 M malonate	93	108	97	96
11	Same	96	114	114	115
12	20 cc. 1 M malonate	106	110	121	123
13	Same	84	109	102	135
14	10 cc. 1 M malonate at 0 hr., 10 cc. at 1 hr.	110	99	212	107

\* Convulsions.

which received twice as much malonate as Rabbits 10 and 11, showed a definite rise.

The true meaning of this antagonism between insulin and malonate *in vivo* is not clear, since a proper interpretation depends upon what insulin does in the living animal. If the decrease in blood sugar following administration of insulin is entirely due to withdrawal of sugar into liver storage, this phenomenon would appear to concern an aspect of insulin activity different from its effect in increasing the oxygen consumption *in vitro*. Nevertheless it is curious that malonate and insulin should counteract each other both *in vitro* and *in vivo*. Since the action of malonate *in vitro* presumably concerns the C<sub>4</sub> acids, it is likely that these acids are also somehow involved in the action of insulin *in vivo*.

*Insulin in Other Species*—Since most of the experiments with insulin *in vitro* have been carried out with pigeon muscle, it should be pointed out that this animal exhibits certain peculiarities. The pigeon normally has a high blood sugar content and removal of 95 per cent of the pancreas does not materially alter this value. Furthermore the pigeon is able to tolerate tremendous doses of insulin. We have given up to 150 units within 24 hours without killing the bird, and the blood sugar was reduced only 50 per cent. The apparent recovery of depancreatized birds after 4 weeks might indicate either the presence of an auxiliary respiratory mechanism or the ability to regenerate islet tissue in some other organ. Thus results with pigeon muscle can be applied to mammals only with caution. Hence, our basic experiments *in vitro* were repeated on rabbit heart, rabbit skeletal muscle, and chicken breast muscle. With these more slowly respiring tissues, the results were qualitatively similar, though quantitatively less spectacular than with the more rapidly respiring pigeon breast muscle (4).

#### SUMMARY

Our results support the general conclusion that insulin is a factor in the respiration of muscle. Other necessary factors are the di(tri)-carboxylic acids, cocarboxylase, and substances in muscle juice. Insulin added to normal pigeon breast muscle *in vitro* increased respiration about 20 per cent both in the presence and absence of other supplements; the sensitivity to insulin was in-

creased to 60 per cent by removal of the pancreas. The optimum response was obtained 1 to 2½ weeks after pancreatectomy.

Insulin prolonged respiration, the effect being most pronounced after 2 hours. In muscle from depancreatized birds, insulin also increased respiration. The R.Q. was maintained at normal levels for 4 hours by insulin alone or insulin plus other supplements. Insulin preparations inactivated by heat or alkali failed to stimulate muscle respiration. Insulin action was inhibited *in vitro* by malonate, and insulin and malonate appeared to counteract each other to a certain extent in lowering blood sugar in the rabbit.

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# THE GONADOTROPIC HORMONE OF URINE OF PREGNANCY

## II. CHEMICAL STUDIES OF PREPARATIONS HAVING HIGH BIOLOGICAL ACTIVITY\*

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In an earlier report (1) we have described two methods for obtaining highly active preparations of gonadotropic hormone from early pregnancy urine. By subsequent purification of the most active products obtained in this manner, hormone fractions containing 4000 minimal effective doses<sup>1</sup> per mg. were prepared. Such preparations have been found to be practically homogeneous with respect to certain physical measurements (Paper III) so that we consider them to be the hormone in a high state of purity.

It is proposed here to report upon the preparation of material containing 4000 minimal effective doses per mg. and to give the analytical data of, and the nature of the carbohydrate in, several of our gonadotropic hormone preparations.

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<sup>1</sup> For definition of the minimal effective dose (Friedman's rabbit ovulating unit) see Paper I (1). A preliminary examination of the international standard for the gonadotropic substance of human urine of pregnancy (chorionic gonadotropin) has been made. This examination shows that 1 minimal effective dose in this laboratory requires about 160 micrograms of the international standard. Since 1 international unit is defined as 100 micrograms of international standard, 1 minimal effective dose is therefore equivalent to about 1.6 international units in our experiments. A preparation assaying 4000 minimal effective doses per mg. would, therefore, assay over 6000 international units per mg.

## EXPERIMENTAL

*Preparation of Purified Hormone*—0.100 gm. of a preparation obtained as previously reported (1) and assaying 3000 minimal effective doses per mg. was dissolved in 2 cc. of ice-cold water. The pH was adjusted to approximately 5 with 0.1 N acetic acid and the solution vigorously shaken at 0–5° for 2 hours with 0.5 cc. of chloroform. After centrifugation in the cold, the upper aqueous layer was separated and the chloroform emulsion washed by shaking for several minutes with 0.5 cc. of water. After centrifugation, the combined clear aqueous solutions were dialyzed in a cellophane bag against approximately 1 liter of distilled water for 24 hours at 0–5°. The solution within the bag was then poured into 30 to 40 cc. of cold dry acetone and a trace of sodium acetate added to induce flocculation. After chilling for several hours, the flocculent precipitate was centrifuged, washed once with cold absolute alcohol, then several times with cold acetone, and finally dried *in vacuo*. 0.080 gm. of a product (Preparation 214D) assaying 4000 minimal effective doses per mg. was obtained in this manner.

*Carbohydrate of Pregnancy Urine Gonadotropin*—Since our most highly purified gonadotropic material contains carbohydrate, we naturally wished to determine its amount. For this purpose, the Hagedorn-Jensen and Shaffer-Hartmann methods were first used. As the results of the former method were higher than those of the latter, and, as the former method is known to yield high results in the presence of the hydrolytic products of protein, it was assumed that the values given by the Shaffer-Hartmann method were more nearly correct. Still lower results were obtained with the Shaffer-Hartmann method after clarification with zinc hydroxide. On account of the discrepant results obtained by the various methods used, an independent procedure was sought for. Accordingly the orcinol method (2) as modified by Sørensen and Haugaard (3) was investigated.

This colorimetric method yields, to some extent, colors which are different with different sugars, so it was necessary to determine the identity of the sugar in the gonadotropin. Therefore, solutions of various sugars were studied. Since uronic acid, pentose, and ketohexose had previously been shown to be absent from our hormone preparations, the common aldohexoses, glucose, galactose, and mannose, and equimolar mixtures of any two of these

sugars, were examined. Although the rate of color development was studied in each case according to the procedure of Sørensen and Haugaard, the results, in agreement with the report of Hartmann and Benz (4), were not adequate to warrant a final decision concerning the nature of the carbohydrate in the hormone. Ultimately it was found advisable simply to study by means of the Evelyn photoelectric filter photometer the colors developed after heating 20 minutes at 80–81°; filters exhibiting maximum transmission at 420 and 520  $m\mu$  were used. In Table I are listed the ratios of the extinction coefficients  $\epsilon_{420}/\epsilon_{520}$  determined for the sugars, the mixtures of sugars, and the hormone.<sup>2</sup> Each value represents the average of five or more individual determinations. (Glucose was not studied because it had been excluded by the method described below.) Information summarized in Table I permits the conclusion that the sugar of the hormone does not consist of mannose, or equimolar mixtures of glucose and mannose, or of glucose and galactose.

A simultaneous study of the carbazole method of Dische (5) showed that this procedure, when modified, yields more consistent and conclusive results. The details of the study of this procedure have been recorded elsewhere (6). From the relative transmissions obtained with this method, the ratios,  $\epsilon_{520}/\epsilon_{420}$ , were calculated. The ratios obtained by using a number of hormone preparations together with the ratios previously obtained with various sugars are given in Table II. A comparison of the figures shows that the sugar of the purified hormone is not glucose, mannose, or an equimolar mixture of glucose and galactose. With both the orcinol and carbazole methods the averages of ratios given by the hormone preparations are somewhat closer to the ratio of galactose than to that of an equimolar mixture of galactose and mannose. Furthermore, we feel that the uncontrollable errors of the carbazole method are more likely to cause the hormone ratio to be low rather than high when this method is used (6). Aqueous solutions of these preparations are faintly yellow in color and absorb light sufficiently at 420  $m\mu$  to increase the error in the same direction; namely, to make the apparent ratio  $\epsilon_{520}/\epsilon_{420}$  lower than the true one. These considerations lead us to conclude that the sugar of the hormone is galactose (8). Further support

<sup>2</sup>  $\epsilon_{420}$  refers to  $\epsilon_0$  (the negative log of the transmission) at 420  $m\mu$ , etc. The internal diameter of the test-tubes employed was 19 mm.



for this conclusion has been gained by the isolation of galactose from two crude preparations and by the inability to isolate mannose in one instance. We feel that our conclusion is probably correct but that further proof obtained by the isolation of the sugar from more highly purified preparations is desirable.

*Isolation of Galactose*—0.20 gm. of a crude gonadotropin preparation assaying 500 minimal effective doses per mg. was hydrolyzed with 25 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  at  $100^\circ$  for 6 hours. To the cooled solution 10 cc. of Hopkins' reagent (10 per cent  $\text{HgSO}_4$  in 5 per cent  $\text{H}_2\text{SO}_4$ ) were added, with stirring, followed by the addition of enough saturated  $\text{Ba}(\text{OH})_2$  to render the mixture only faintly acidic. Excess solid  $\text{BaCO}_3$  was then introduced, and stirring was continued for several hours. 5 volumes of alcohol were added, the mixture allowed to stand in the cold room overnight, and,

TABLE I  
*Comparison of Colors Produced by Hormone and Various Hexoses in Orcinol Reaction*

Concentrations of sugar of 0.1 mg. per cc. were used.

Preparation	$\epsilon_{420}/\epsilon_{520}$
Mannose.....	5.90
Glucose-mannose.....	5.86
Hormone (2000-3000 minimal effective doses per mg.)....	4.74
Galactose.....	4.67
Galactose-mannose.....	4.45
Glucose-galactose.....	4.37

after filtration, the precipitate was washed repeatedly with 95 per cent alcohol. The combined filtrate and alcohol washings were evaporated to dryness *in vacuo*. The residue was dissolved in 2 cc. of water, 6 cc. of absolute alcohol were added, and the cloudy suspension chilled 5 to 6 hours. After centrifugation, the supernatant liquid was again evaporated to dryness and the residue extracted with 3 to 4 cc. of warm methyl alcohol. Upon evaporation of the alcoholic solution a small microcrystalline residue was obtained.<sup>3</sup> The material was dissolved in 0.5 cc. of water and

<sup>3</sup> 1 gm. of another preparation containing 1000 to 2000 minimal effective doses per mg. was studied in this way. In this instance, the corresponding residue was dissolved in 0.5 cc. of water and about 0.02 cc. was tested with carbazole which yielded a color giving a ratio of  $\epsilon_{520}/\epsilon_{420} = 1.0$ . Mannose phenylhydrazone could not be obtained from the remainder of this solu-

treated with 0.1 gm. of freshly distilled  $\alpha$ -methylphenylhydrazine. Alcohol was added dropwise until a clear solution was obtained.

TABLE II  
*Comparison of Colors Produced by Several Hormone Preparations in Carbazole Reaction*

Preparation No.	Activity	Concentration	$\epsilon_{320}$	$\epsilon_{420}$	$\epsilon_{320}/\epsilon_{420}^\dagger$
	<i>m.e.d.* per mg.</i>	<i>mg. per cc.</i>			
85C	2000	0.98	0.321	0.310	1.04
160C‡	2000	0.56	0.134	0.140	0.96
195C	2000	0.98	0.294	0.312	0.94
198C	2000	0.99	0.357	0.330	1.08
209C	2000	1.08	0.283	0.311	0.91
214C	3000	0.98	0.270	0.264	1.02
214C	3000	0.66	0.218	0.220	0.99
101C	3000	0.93	0.348	0.346	0.99
82D§	3000	0.94	0.341	0.324	1.05
83D	3000	1.01	0.346	0.326	1.06
85D	3000	0.99	0.316	0.315	1.00
99D	4000	1.22	0.426	0.458	0.93
101D	4000	1.18	0.426	0.432	0.99
214E	4000	1.15	0.409	0.432	0.95
214F¶	4000	0.65	0.231	0.229	1.01

\* Minimal effective doses.

† The ratios in the last column, which are averages of duplicates agreeing within 5 per cent, are to be compared with the ratios obtained recently with various hexoses and their equimolar mixtures: glucose 2.60 to 2.70, galactose 1.10 to 1.20, mannose 0.65 to 0.70, galactose-mannose 0.80 to 0.85, glucose-mannose 1.10 to 1.20, glucose-galactose 1.60 to 1.70. These values are slightly higher than those previously reported (6), since new carbazole reagents have been employed and traces of nitrate rigorously excluded.

‡ All of the C preparations were made by the method previously described (1).

§ The D preparations were obtained from the corresponding C fractions by the procedure described in the experimental part of this paper.

|| Prepared from Preparation 214C by precipitation with tannic acid and subsequent removal of the tannic acid.

¶ Prepared from Preparation 214C by precipitation with  $I_2$  and KI (7).

This was allowed to stand 10 to 15 hours at room temperature, during which period characteristic crystals of galactose methyl-

tion. These observations confirm our belief that galactose is the only sugar present.

phenylhydrazone were deposited. After subsequent chilling and centrifugation the product was recrystallized from a minimal amount of 30 per cent alcohol. Approximately 5 mg. of product were obtained, melting at 187°. A mixed melting point with an authentic sample of galactose methylphenylhydrazone was 187–188°.

In an attempt to isolate mannose 0.5 gm. of a similar preparation was hydrolyzed in the same manner. The fractionation was carried out essentially as described above. From a volume of 0.5 cc. of aqueous solution no precipitate of mannose phenylhydrazone could be obtained after 24 hours. Addition of methylphenylhydrazine to the solution produced after 8 to 10 hours characteristic crystals of galactose methylphenylhydrazone. Mannose appears to be absent or, at the most, present only in traces in these preparations.

*Composition of Purified Hormone Preparations*—The data of one of our purest preparations are given in Table III, and, for comparison, the results of analyses of a number of less pure preparations possessing biological activities ranging from 500 to 3000 minimal effective doses per mg. All preparations were found to be remarkably similar in composition, despite an 8-fold difference in activity. The purest fractions contained, on an ash-free basis, approximately 50 per cent carbon, 7 per cent hydrogen, 12 per cent nitrogen, and 2 per cent sulfur (most of which was probably inorganic). There was no phosphorus present. Uronic acid, pentose, and ketohexose likewise were absent. Arginine and histidine were absent or present only in traces.

The quantitative estimations of galactose were carried out by means of the carbazole reaction (6), with a filter transmitting at 540 m $\mu$ . Values of 10 to 12 per cent galactose were obtained (Table III). The hexosamine values ranged from 5 to 6 per cent.

The Shaffer-Hartmann method which was used for determining the total reducing sugar was standardized with galactose and the results calculated in terms of that sugar. The accuracy of this method, when used on solutions such as ours, is confirmed by the fact that the sums of the galactose (carbazole method) and the hexosamine values approximate closely in most cases the Shaffer-Hartmann (with zinc hydroxide) figures. In most cases the ratio of total reducing sugar (calculated as galactose) to hexosamine was approximately 3:1. The molar ratio of galactose to hexos-

amine was approximately 2:1, thus suggesting that the polysaccharide may be comprised of hexosamine digalactose units. Since the molar ratio of acetyl to hexosamine in all cases was 2:1, it is likely that the amino group of the hexosamine moiety

TABLE III  
*Composition of Gonadotropic Hormone Preparations*

All analyses are calculated on an ash-free basis. The samples were dried *in vacuo* at 90–100°.

Preparation No. ....	165C	160C	195C	198C	209C	214C	214D
Activity, <i>m.e.d.</i> per mg. ....	500	2000	2000	2000	2000	3000	4000
Ash, % .....	11.10	10.40	6.95	5.00	3.28	10.85	1.92
C, % .....						50.06	50.42
H, % .....						7.03	6.95
N, % .....	11.81	11.70	12.85	12.25	12.76	12.35	12.03
S, % .....			3.14	3.06	2.54	2.72	1.96
Initial sugar, % ..		6.1	5.4	7.3	5.3	5.6	6.6
Total sugar, % ..	17.9	18.8	18.4	19.4	18.0	19.5	17.8
“ “ † % ..		16.2	15.5		15.2	16.0	16.2
Galactose, ‡ % ..		10.0	10.8	12.4	10.3	11.8	10.7
Hexosamine, § % ..	5.22	4.95	4.90	5.79	5.38	6.32	5.2
Acetyl, ¶ % .....	2.37	2.66	2.76	2.95	2.90	3.01	
Molar ratio							
Acetyl to hexosamine .....	1.87	2.23	2.32	2.14	2.24	1.96	
Galactose to hexosamine ..		2.1	2.2	2.1	1.9	1.9	2.1

\* Initial and total reducing sugar was estimated by the Somogyi (9) modification of the Shaffer-Hartmann method after preliminary hydrolysis with 2 N HCl for 2 to 3 hours at 100°.

† Clarification with Zn(OH)<sub>2</sub>.

‡ Carbazole method.

§ Palmer-Smyth-Meyer (10) modification of the Elson-Morgan procedure.

|| We thank Dr. K. Meyer for these analyses.

¶ Suzuki (11) modification of the Friedrich, Rappaport, and Sternberg method.

is acetylated, and that the other acetyl group is attached to some other portion of the hormone molecule. That the amino group of the hexosamine is acetylated is further indicated by hydrolysis experiments (Table IV). Although heating at 100° for 2 to 3 hours with 2 N HCl was sufficient to liberate all of the available

reducing groups, maximum hexosamine values could not be obtained until the hormone was heated for 8 to 10 hours with 4 N HCl. As hexosamine gives a color with the reagents only when the amino group and the reducing group are free, the experiment just described may be explained on the assumption that acetyl is split off slowly.

The presence of galactose and hexosamine suggested that the carbohydrate of the hormone might be serologically related to the blood Group A specific polysaccharide, as was found to be the case by Meyer and coworkers (12) for their neutral polysaccharide of gastric mucosa and by Goebel (13) for the carbohydrate of

TABLE IV  
*Liberation of Reducing Sugar and Hexosamine. Hydrolysis of Hormone with 2 N HCl at 100°*

Period of hydrolysis	Reducing sugar	Hexosamine*
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>
0	5.9	0.4
2	18.0	2.1
4	17.9	3.3
8		4.4
10	17.6	4.4†

\* Palmer-Smyth-Meyer (10) modification of the Elson-Morgan procedure.

† Maximum values of 5.4 per cent were not obtained until the hormone was heated with 4 N HCl for 8 to 10 hours.

peptone. In a dilution of 1:1000, Preparation 195 (2000 minimal effective doses per mg.) failed to develop a precipitate with Group A antisera nor did the solution inhibit hemolysis of sheep cells in anti-A rabbit serum.<sup>4</sup> The carbohydrate portion of the hormone molecule appears therefore to be unrelated serologically to the blood Group A specific polysaccharide.

#### DISCUSSION

The similarity in the chemical composition of our gonadotropic preparations which vary markedly in biological potency suggests the possibility that the less pure preparations contain significant

<sup>4</sup> Through the courtesy of Dr. W. Henle.

amounts of inactivated hormone, or that they contain as a contaminant another urinary glycoprotein of similar composition. The fact that considerable inactivation occurs in aqueous solution at low temperatures (1) argues for the formation in urine (as well as in solution during the fractionation process) of degradation products having chemical and physical properties very similar to those of the hormone. If this decomposition is accompanied by the loss or rupture of certain portions of the molecule, it cannot be detected by the analytical methods used.

Our inability to improve by chemical means the potency of occasional preparations made by our process, but assaying 1000 minimal effective doses per mg., lends further support to our belief that the urine may contain inactivated hormone and that a continuous degradation is proceeding from the time of collection of the urine to the preparation of our most active products. Furthermore, the fact that in some instances material containing 4000 minimal effective doses per mg. may be obtained, whereas at other times somewhat less active preparations are recovered under identical conditions, strengthens this belief.

Unsuccessful attempts have been made to isolate the polysaccharide of our hormone preparations by treatment with 2 per cent  $\text{Na}_2\text{CO}_3$  at  $70^\circ$  and  $100^\circ$  for 10 minutes (12) or by boiling with 10 per cent  $\text{Ba}(\text{OH})_2$  for 24 hours. Further efforts will be made after sufficient quantities of purified hormone are available to us.

We wish to acknowledge the assistance of Miss Dorothy B. Hood.

#### SUMMARY

Further details of methods for purification of the gonadotropin of human pregnancy urine are presented, along with analytical data of a number of preparations thus purified. The analytical data suggest that these preparations are glycoproteins.

A study of the hormone by means of the orcinol and carbazole reactions indicates that the non-hexosamine carbohydrate is galactose. This conclusion has been confirmed by the isolation of galactose from hormone preparations. Attempts to isolate mannose have been unsuccessful.

The molar ratio of galactose to hexosamine (2:1) suggests the

possibility that the carbohydrate portion of the hormone molecule is built up of hexosamine digalactose units.

Evidence is presented suggesting that the amino group of the hexosamine is acetylated and another acetyl group is attached elsewhere in the molecule.

The hormone appears to bear no serological relationship to the blood Group A specific polysaccharide.

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# THE GONADOTROPIC HORMONE OF URINE OF PREGNANCY

## III. EVIDENCE OF PURITY OBTAINED BY STUDIES OF ELECTROPHORESIS AND SEDIMENTATION\*

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In Paper II (1) of this series we reported upon the chemical composition of purified preparations of pregnancy urine gonadotropin. These analyses confirmed and extended the investigations of Fischer and Ertel (2) and of Meyer (3), and supported their belief that pregnancy gonadotropin is a glycoprotein. Recently, we have also reported (4) in a preliminary way the results of a study of some physical properties of purified preparations of the hormone. The purpose of the present communication is to record these studies in further detail, and to discuss their significance in relation to the purity of such preparations.

### EXPERIMENTAL

*Electrophoresis Studies*<sup>1</sup>—Two highly active hormone preparations were investigated by means of the Tiselius electrophoresis apparatus. Although both preparations were similarly made (the details have been described elsewhere (1, 5)), one preparation was less active than the other. The less active preparation (No. 219D, assaying 2000 to 3000 minimal effective doses<sup>2</sup> per mg.) was employed in the first experiment, after a preliminary dialysis

\* We wish to thank Parke, Davis and Company for financial aid.

<sup>1</sup> We wish to acknowledge our gratitude to Dr. Florence B. Seibert who carried out the electrophoresis experiments for us.

<sup>2</sup> The biological test method employed (6) has been discussed elsewhere (1, 5).



for 24 hours against phosphate buffer having a pH of 7.0 with an ionic strength of 0.1. The final solution of hormone had a volume of 10 cc. and a concentration of 1.25 per cent. 200 volts (15 milliamperes) were employed. Two mobile components were observed; *viz.*, one component having a mobility of  $4.32 \times 10^{-5}$  sq. cm. sec.<sup>-1</sup> volt<sup>-1</sup>, and another, in much lower concentration, with a mobility only slightly greater than the first. A moderately strong immobile band (presumably due to polysaccharide) was likewise observed. Soon after the voltage was increased to 300, decomposition appeared to set in, and the experiment was therefore stopped (2½ hours after the beginning). The solutions in the various compartments were recovered in the customary way. The materials in these solutions were isolated, after dialysis against distilled water at 0–5° for 15 hours, by precipitation with 20 volumes of cold acetone. From the compartment containing only the mobile fraction, 25 mg. of an active product were recovered which assayed 1000 to 1500 minimal effective doses per mg.<sup>3</sup> Upon analysis, this preparation was found to contain 17.8 per cent total reducing sugar (Shaffer-Hartmann method), 5.98 per cent hexosamine, and 12.3 per cent galactose (by the carbazole method (1, 7)), values similar to those obtained before electrophoresis. The ratio  $\epsilon_{520}/\epsilon_{420}$ , determined by the carbazole procedure (7), was found to be 1.05, which is interpreted as confirmatory of previous evidence that galactose is the only hexose present (1). This product gave positive biuret and ninhydrin tests.

From the compartment containing the immobile component, 1 to 2 mg. of solid was recovered. The material, presumably polysaccharide, was non-dialyzable, and contained sugar. Since there was so little material available, no quantitative estimation of sugar was made.

From this experiment it seems clear that, while preparations containing 2000 to 3000 minimal effective doses per mg. are impure, they nevertheless appear to contain as impurity but one other protein contaminant in significant amounts, along with a small

<sup>3</sup> The reduced activity of the resulting product was in all probability due to decomposition which occurred during the experiment. The conditions of the latter required solution of the hormone in water over a period of 48 hours, and this has been found (5) to cause significant loss of biological activity. See also foot-note 6.

quantity of polysaccharide. Because of the similar electrochemical characteristics of the two protein compounds, we believe that one represents a degradation product of the hormone.

Preparation 214D (4000 minimal effective doses per mg.) was also investigated in the Tiselius apparatus. A concentration of 0.5 per cent was employed, with the conditions of the experiment maintained as described above except for a slight decrease in the voltage used (180 volts). In this case only one mobile band ap-



FIG. 1. Photographs obtained with Preparation 214D in the Tiselius electrophoresis apparatus. Exposures 1 and 2 represent the mobile protein band after 28 and 60 minutes respectively. Exposure 3 shows the protein band pushed down by the compensator 2 hours after the start. Exposure 4 shows the immobile carbohydrate component.

peared, as can be seen in Exposures 1 and 2 of Fig. 1,<sup>4</sup> taken 28 and 60 minutes respectively after the start of the experiment. After 2 hours there could still be observed but a single mobile band (Exposure 3). A very faint immobile band was observed at this time (Exposure 4), indicating the presence of a trace of

<sup>4</sup> The photographs were made by means of the Toepler schlieren method (8). This is also true of the experiments in which the ultracentrifuge was used.

uncharged contaminant (presumably polysaccharide). In view of the small amount of immobile component isolated in the first experiment, we believe that only traces of a similar impurity were present in the more highly purified material.

The mobility was found to be  $4.85 \times 10^{-5}$  sq. cm. sec.<sup>-1</sup> volt<sup>-1</sup>, a value which agrees well with that obtained in the first experiment. It seems clear from this experiment that Preparation 214D is practically homogeneous with respect to electrical charge.

*Ultracentrifuge Studies*<sup>5</sup>—A 1.8 per cent solution of Preparation 214D (4000 minimal effective doses per mg.) in water showed one single sedimenting band when centrifuged at 250,000 to 260,000 times gravity. Exposures 1 to 4 (Fig. 2) show the sedimentation

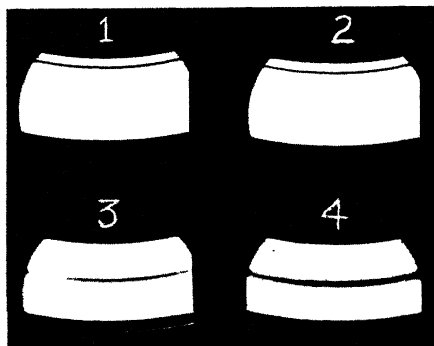


FIG. 2. Photographs obtained with Preparation 214D in the ultracentrifuge. Exposures 1 to 4 were taken during a time interval of  $1\frac{1}{2}$  hours.

during a time interval of  $1\frac{1}{2}$  hours. The sedimentation constant was found to be  $4.3 \times 10^{-13}$  cm. sec.<sup>-1</sup> dyne<sup>-1</sup>, uncorrected for temperature. Unfortunately, during a part of the run the speed of the centrifuge was varied and therefore this value can only be an approximation.

A second preparation (4000 minimal effective doses per mg.) obtained by precipitation with I<sub>2</sub> in KI (Meyer (3)) was similarly examined. In this case a 0.2 per cent solution was employed. As in the previous experiment, only a single sharp band was ob-

<sup>5</sup> We wish to thank Dr. A. E. Severinghaus and Dr. J. A. Chiles, Jr., for making the measurements reported.

served.<sup>6</sup> The sedimentation constant was found to be  $5.4 \times 10^{-13}$  cm. sec.<sup>-1</sup> dyne<sup>-1</sup>, uncorrected for temperature. If the molecule is spherical, these values indicate a molecular weight of 60,000 to 80,000.

*Isoelectric Point*—The isoelectric point of a number of preparations ranging in activity from 2000 to 4000 minimal effective doses per mg. was determined by means of a Bull microelectrophoresis apparatus (9). Each preparation was dissolved in 0.02 M citrate buffer and 0.1 and 0.01 per cent solutions of the hormone were mixed with small quantities (0.1 volume) of an aqueous suspension of colloidal collodion prepared by the method of Zozaya (10). The suspensions were stirred for 30 minutes. The pH of the buffer solutions and the suspensions (after stirring) was checked

TABLE I  
*Charge on Collodion Particles after Adsorption of Hormone*

Preparation No.	Activity	pH						
		4.96	4.10	3.50	3.30	3.20	3.05	2.25
	<i>m.e.d.* per mg.</i>							
214D	4000	—	—	—	—	+	+	+
198C	2000	—	—	—	—	±	+	+
160C	2000	—	—	—	—	+	+	+

\* Minimal effective doses.

by means of a glass electrode. In every case (see Table I) the isoelectric point was found to lie between pH 3.2 and 3.3. Spielman and Meyer (11) have reported electrophoresis experiments that indicate an isoelectric point of 3.3 to 3.5.

#### DISCUSSION

If the implications of the work of Fischer and Ertel, of Meyer, and of ourselves, relative to the chemistry of pregnancy gonado-

<sup>6</sup> These solutions of hormone which appeared homogeneous in the ultracentrifuge were allowed to stand for approximately 36 hours in the cold. Upon reexamination in the ultracentrifuge six distinct sedimenting components were observed (personal communication of Dr. A. E. Severinghaus). It is clear that extensive decomposition takes place under these conditions.

tropin are valid, this hormone is a glycoprotein containing a high percentage of carbohydrate. Since, to our knowledge, no glycoprotein having a similarly high content of sugar has yet been obtained in crystalline form, it is not surprising that our attempts to crystallize the hormone have failed. Despite such failure, however, we believe that our preparations which assay 4000 minimal effective doses in the postpartum rabbit contain the hormone in a high state of purity.

The order of the biological activity of our best preparations is constantly in the range of 4000 minimal effective doses per mg., irrespective of the methods of purification by which they have been obtained. We have not been able to raise the potency of such preparations by further treatment. The chemical analyses of products of varying biological activity indicate that, with increase of activity toward the maximum, there is little change in the content of hexosamine, acetyl, and galactose. The impurities in the less active preparations therefore would seem to be similar in composition to the active substance and may be degradation products, already preformed in pregnancy urine or formed during the course of the fractionation procedures.

It seems evident from the electrophoresis experiments reported in this paper that the biological activity is associated with mobile material which gives the biuret and ninhydrin tests and contains 12 per cent galactose. We have reported elsewhere (1) our inability to separate the carbohydrate from the protein by dialysis or by heating with dilute alkali. Similar findings have been reported recently by Hartmann and Benz (12). No evidence of separation could be obtained by shaking aqueous solutions of the hormone with chloroform (13) at a pH range of 5 to 8. The linkage between carbohydrate and protein is therefore a firm one. This evidence together with the analytical data indicates that Preparation 214D is a glycoprotein. It contains 4000 minimal effective doses per mg. and, with the method used, appears to be practically homogeneous with respect to electric charge.

From the results of the ultracentrifuge studies it also seems clear that Preparation 214D and another preparation of similar biological activity (prepared by precipitation with iodine) are homogeneous with respect to molecular weight. Although the sedimentation values here reported are admittedly approximations,

it would appear likely that the minimum molecular weight of the hormone lies somewhere between 60,000 and 80,000. An accurate estimation of molecular weight will necessitate a determination of the diffusion constant, but because of the marked instability of the gonadotropin in aqueous solution such a study has not yet been possible. The presumably large molecular weight seems very surprising in view of the fact that the hormone is excreted by the normal human kidney. However, the actual weight of hormone excreted into the urine is quite small.

It is realized that additional evidence with respect to homogeneity is desirable. We hope that, as more material becomes available, it will be possible to carry out more extensive experiments both with the ultracentrifuge and with the Tiselius apparatus.

It should be observed that the acid isoelectric point of the hormone is probably a reflection of the low content of basic amino acids. It is therefore not surprising that arginine and histidine are absent, or present only in traces. The lysine content has as yet not been investigated.

#### SUMMARY

Electrophoretic studies of a pregnancy urine gonadotropin preparation containing 4000 minimal effective (ovulating) doses per mg. show that it is a protein very nearly electrochemically homogeneous, with a mobility of  $4.8 \times 10^{-5}$  sq. cm. sec.<sup>-1</sup> volt<sup>-1</sup> at pH 7. The characteristic biological activity of the hormone is associated with this mobile protein and analytical data indicate that it is a glycoprotein.

In the ultracentrifuge, the same preparation as well as a second one of equally high biological activity but purified by a different method sedimented as a single component. They appear therefore to be homogeneous with respect to molecular weight. The calculated sedimentation constant (approximately  $5 \times 10^{-13}$  cm. sec.<sup>-1</sup> dyne<sup>-1</sup>) suggests that the minimal molecular weight lies between 60,000 and 80,000.

The isoelectric point was found to be pH 3.2 to 3.3.

The evidence here reported indicates that hormone preparations assaying 4000 minimal effective doses per mg. are practically homogeneous glycoproteins.

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## NOTE ON THE ACTION OF COPPER AND PHENYL-HYDRAZINE ON CERTAIN DEHYDROGENASES

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The inhibitory action of copper on succinoxidase is the result of its catalytic effect on the oxidation of —SH groups in the enzyme protein (1). It follows from this that a concentration of copper which causes no inhibition immediately after its addition to the enzyme should cause an inhibition if shaken with it in air. This has been shown to be the case. Because copper catalyzes the oxidation of a number of different groups, it was of interest to see whether it had a similar effect on other enzymes.

### EXPERIMENTAL

Rat liver was used as the source of the enzymes. It was treated as already described (2) with the exception that the insoluble proteins were washed with phosphate buffer at pH 7.8 instead of pH 6.7. After four washings by centrifugation, a light yellow suspension was obtained which was almost completely free of hemoglobin and oxidizable substrates. If the  $Q_{O_2}$  of the succinoxidase is taken as 100 per cent, then the concentration of the other enzymes present was as follows: cytochrome-cytochrome oxidase, measured by the rate of oxidation of *p*-phenylenediamine, 95 per cent; choline oxidase, 57 per cent; *D*-amino acid oxidase, with proline as substrate, 42 per cent; amine oxidase, with isomylamine as substrate, 11 per cent. These values were obtained with saturation concentrations of substrate and were constant from one preparation to another.

Because cupric ion alone oxidizes *p*-phenylenediamine, its effect on the cytochrome oxidase could not be studied. For the other enzymes the following procedure was used. 0.5 cc. of the sus-



pension was added to the Warburg vessels with enough 0.05 M phosphate buffer, pH 7.8, to make the final volume to 2.0 cc.  $\text{CuSO}_4$  was added directly to the mixture and followed either immediately or after a certain time of shaking at  $37^\circ$  by 2.0 mg. of the appropriate substrate. The results showed that shaking for 1 hour before the substrate was added always increased the inhibition of the succinoxidase but had no effect on the inhibition of the other enzymes (Table I). In other words, there are no groups in the other enzymes susceptible to catalytic oxidation by copper under conditions which are favorable to the catalytic oxidation of the sulfhydryl groups in the succinoxidase. As shown in Table II, these enzymes can be inhibited by copper but

TABLE I

*Effect of  $1 \times 10^{-4}$  M  $\text{CuSO}_4$  When Added Immediately before Substrates and after Being Shaken with Enzymes in Air for 60 Minutes before Addition of Substrates (pH 7.8, Temperature  $37^\circ$ )*

The figures are given in percentage activity. The controls without copper are given the value of 100 per cent even when, as in the case of the *d*-amino acid oxidase, there is a marked decrease in activity.

Oxidase	Substrates added immediately	Substrates added after 60 min. shaking in air
	<i>per cent</i>	<i>per cent</i>
Succinic.....	92	51
Choline.....	100	96
<i>d</i> -Amino acid.....	54	55
Amine.....	85	83

the inhibition is a function only of the copper concentration and not of time. This indicates that the copper is combining with some groups in the enzymes. From a comparison of the relative inhibitions produced by different concentrations of copper on the various enzymes with those produced by phenylhydrazine under the same conditions (see Table II) it is obvious that the concentration of the enzymes present does not determine the relative sensitivities to the two substances. Thus the amine oxidase which is present in the lowest concentration is much less sensitive to copper than the other enzymes but much more sensitive to phenylhydrazine.

Control experiments to test the effect on the enzyme activities of shaking for 1 hour at 37° without copper revealed one interesting

TABLE II

*Effect of Different Concentrations of CuSO<sub>4</sub> and Phenylhydrazine on Percentage Activity of Certain Oxidases in Standard Rat Liver  
Preparation at pH 7.8 and 37°*

The substrates were added immediately after the addition of the copper and phenylhydrazine.

	Concentration	Oxidase				
		Choline	<i>d</i> -Amino acid	Amine	Succinic	Cytochrome
	$M \times 10^{-4}$	per cent	per cent	per cent	per cent	per cent
CuSO <sub>4</sub>	0.5	100	100	90	100	
	1.0	100	48	85	86	
	1.5	60	0	83	50	
	2.0	47	0	79	32	
	2.5	32	0	71	4	
Phenylhydrazine	1.0	55	99	46	100	100
	2.3	25	86	24	100	90
	4.5	11	75	0	27	80
	7.5	0	58	0	0	60

TABLE III

*Effect of Shaking for 60 Minutes at 37° and pH 7.8 in Different Oxygen Tensions on Percentage Activity of Enzymes*

The values when the substrates were added immediately are designated 100 per cent. The value for the *d*-amino acid oxidase with substrate was obtained by the addition of more substrate after 60 minutes.

Oxidase	20 per cent O <sub>2</sub>	2 per cent O <sub>2</sub>
	per cent	per cent
<i>d</i> -Amino acid alone.....	32	72
“ “ with substrate.....	93	
Succinic.....	92	
Choline.....	89	
Amine.....	94	

fact (Table III). Shaking in air under the experimental conditions had little effect on the activity of the succinic, choline, or amine

oxidases but inhibited the *d*-amino oxidase by 50 to 70 per cent. This inhibition occurred in all the preparations of liver and kidney *d*-amino acid oxidase tried and could be markedly decreased if nitrogen with 2 per cent oxygen was substituted for air. The presence of substrate also prevented the inhibition. This indicates that the *d*-amino acid oxidase contains a group oxidizable by oxygen but the oxidation cannot be catalyzed by copper.

The copper inhibition of the choline, amine, and *d*-amino acid oxidases, unlike that of the succinoxidase, is not readily reversible. It is not affected by the concentration of substrate nor by changing the pH from 7.8 to 6.7.

The effect of phenylhydrazine on catalase was studied by Blaschko (3). Its effect on other oxidative enzymes does not seem to have been studied. Table II shows the relative inhibitions produced by various concentrations on five enzymes under the same conditions. The amine and choline oxidases are most sensitive. There is no effect on the succinoxidase until a certain concentration of phenylhydrazine is reached, after which the inhibition increases rapidly with concentration. The *d*-amino acid oxidase and the cytochrome oxidase are relatively insensitive. All the inhibitions are reversible by the addition of aldehyde to the mixture and the initial presence of aldehyde can prevent inhibition. Incubation with the enzyme for various times before the addition of substrate does not change the percentage inhibition.

#### DISCUSSION

The purpose of the above experiments was to study the relative effects of the two substances on a group of enzymes under exactly the same conditions, thereby eliminating the effect of any interfering substances that may be present in the standard preparation used. A comparison of this type would not be valid if in an attempt to purify the enzymes different preparations were used, because the possibility of interfering substances cannot be eliminated unless the enzymes can be recrystallized several times. Although no conclusions can be drawn as to the nature of the active groups in the various catalysts, it is probable, because of the low concentrations used, that both substances are causing chemical rather than physical changes in the proteins.

## SUMMARY

By the use of a standard rat liver preparation the relative effects of copper and phenylhydrazine on various oxidative enzymes were studied.

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# THE DETERMINATION OF NEUTRAL FAT GLYCEROL IN BLOOD WITH PERIODATE

## APPLICATION TO THE DETERMINATION OF ARTERIOVENOUS DIFFERENCES IN NEUTRAL FAT

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Recent work (1) on the blood precursors of milk fat, by attaching special significance to the triglycerides of blood in the formation of milk, has illustrated forcefully the need of a more direct method for the quantitative evaluation of neutral fat in blood. Until Kelsey (2) reported the use of lipases, the isolation of the glyceride fatty acids had not been accomplished because of the homologous nature of the fatty acids in the acetone-soluble lipids of blood. The only alternative for the direct estimation of neutral fat is the determination of glycerol after saponification of the acetone-soluble lipids. A method suitable for this purpose is proposed herein. Its application to the determination of arteriovenous differences in neutral fat of the blood of the lactating cow is described.

The method is based on the reaction of periodic acid and periodates on polyhydric alcohols or acids having adjacent hydroxyl groups. This reaction was discovered and its applications described by Malaprade (3). Glycerol reacts with periodate in acid solution according to the equation



The reaction is quantitatively accurate for small quantities of glycerol in water solution and occurs quite rapidly at ordinary temperatures under conditions that do not need to be rigorously defined.

In principle the method involves the isolation of the neutral fat glycerol from the saponified acetone-soluble blood lipids and

treatment of the acidified water solution of glycerol with a measured quantity of periodate reagent and determination of the excess periodate in the reaction mixture after the reaction is complete.

*Determination of Glycerol with Periodate*

The periodate reagent used in the present work was prepared by dissolving 0.6250 gm. of reagent grade  $\text{KIO}_4$  in 500 cc. of 0.1 N  $\text{H}_2\text{SO}_4$ . 10 cc. of this solution, which will oxidize a maximum of 2.5 mg. of glycerol, are quite adequate for the determination of glycerol in blood. Where it is desired to determine larger quantities of glycerol, the concentration of the  $\text{KIO}_4$  in the reagent may be increased to about 4 times that designated above.

For the determination of glycerol 10 cc. of the periodate reagent were pipetted into the water solution of glycerol and 20 to 30 minutes were allowed for the reaction to become complete. The excess  $\text{KIO}_4$  was then determined by the method of Rappaport, Reifer, and Weinmann (4) in which the iodine liberated from periodate in the presence of iodate can be titrated with standard thiosulfate by keeping the pH within the limits of 4.4 to 7. Within this pH range iodine is liberated quantitatively from periodate by KI solution according to the equation



The reaction mixture was neutralized, after the addition of 3 drops of 15 per cent  $\text{MgSO}_4$  solution, by introducing dilute  $\text{NaOH}$  solution dropwise until a faint cloudiness due to  $\text{Mg}(\text{OH})_2$  appeared, after which 0.1 N  $\text{H}_2\text{SO}_4$  was added dropwise until the turbidity disappeared. The reaction mixture was then treated with 10 cc. of phosphate buffer consisting of 12 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and 4.2 cc. of 10 N  $\text{H}_2\text{SO}_4$  in 100 cc. of solution.

2 cc. of 5 per cent KI solution were added and the liberated iodine was titrated with 0.00435 N thiosulfate solution, a potato starch solution being added as indicator near the end of the titration. The difference between this titration value and that of a corresponding blank represents the thiosulfate equivalent of the glycerol oxidized by the  $\text{KIO}_4$ . 1 cc. of 0.00435 N thiosulfate corresponds to 0.5 mg. of  $\text{KIO}_4$  or 0.1 mg. of glycerol.

As alternative procedures for the determination of the excess

periodate, the neutralized reaction mixture can be treated with buffers appropriate for titration of the liberated iodine with standard arsenite solution (5). Fleury and Lange (6) recommend addition of excess standard arsenite and back titration with standard iodine solution, saturated  $\text{NaHCO}_3$  solution being used as the buffer. All of these procedures give essentially the same results.

The validity of the reaction and the limits of sensitivity of the method were established in tests with a water solution of glycerol prepared to contain 0.1 mg. of glycerol per cc. Illustrative results obtained by application of the procedure outlined above to quantities of glycerol from 0.1 to 2.0 mg. are presented in Table I.

TABLE I  
*Determination of Glycerol by Periodate Procedure in Prepared Water Solution*

Glycerol taken	Thiosulfate required for excess $\text{IO}_4^-$	Thiosulfate equivalent to $\text{IO}_4^-$ used	Glycerol found	Glycerol recovered
mg.	cc.	cc.	mg.	per cent
0	24.6			
2.00	4.8	19.8	1.98	99.0
1.50	9.7	14.8	1.48	98.7
1.00	14.6	10.0	1.00	100.0
0.50	19.6	5.0	0.50	100.0
0.20	22.6	2.0	0.20	100.0
0.10	23.7	0.9	0.09	90.0

Further verification of the periodate procedure for the determination of glycerol was obtained with application of the method to triolein and trielaidin. Typical results are shown in Table II. The periodate reagent did not react with the intact triglycerides but after saponification theoretical results could be obtained with or without removal of the fatty acids from the acidified mixture by extraction with petroleum ether.

#### *Determination of Neutral Fat Glycerol in Bovine Blood*

*Alcohol-Ether Extracts*—The phospholipids were precipitated from 100 to 200 cc. of the alcohol-ether extract of plasma (20:1) by the method of Ellis and Maynard (7) and the clear acetone solution, containing the neutral fat, was retained. A volume of



acetone equivalent to this solution was carried through the subsequent procedure as a blank.

The acetone was evaporated and the residue was treated with 10 cc. of redistilled 95 per cent ethanol and 1 cc. of 5 N NaOH. Saponification was effected by heating on the steam bath until the volume was reduced to about one-half. About 10 cc. of water were then added and evaporation was continued until the odor of ethanol could no longer be detected. 1 cc. of 10 N H<sub>2</sub>SO<sub>4</sub> was added to liberate the fatty acids, most of which, along with

TABLE II

*Determination of Glycerol by Periodate Procedure in Triolein and Trielaidin*

Sample and treatment		Triglyceride taken	Glycerol determined	Triglyceride found	
		mg.	mg.	mg.*	per cent
Triolein	Saponified, extracted	4.4	0.46	4.4	100
	“ “	5.6	0.60	5.7	102
	“ “	6.4	0.68	6.5	102
	“ “	7.8	0.78	7.5	96
	“ unextracted	7.8	0.84	8.1	104
	“ “	12.8	1.42	13.6	106
	Unsaponified, “	7.8	0.00		
Trielaidin	“ “	12.8	0.03		
	Saponified, extracted	1.5	0.16	1.5	100
	“ “	2.5	0.26	2.5	100
	“ “	10.0	1.03	9.9	99
	“ unextracted	10.0	1.04	10.0	100
	Unsaponified, “	10.0	0.03		

\* Glycerol  $\times$  9.62.

some cholesterol present, were removed by one extraction with petroleum ether.

The water solution was then cooled in the refrigerator for about an hour and filtered through asbestos in a Gooch crucible. The cooling facilitates filtration, which was most readily accomplished by suction, a test-tube within the suction flask being used as a receiver. The residue was washed several times with water.

The combined filtrate and washings were treated with 10 cc. of the periodate reagent for the determination of glycerol as

described above. Typical results obtained with alcohol-ether extracts of bovine plasma, to some samples of which triolein had been added, are presented in Table III.

*Acetone Extracts*—5 to 15 cc. of bovine blood plasma were pipetted into a 50 cc. centrifuge tube containing 30 cc. of acetone. 10 gm. of anhydrous sodium sulfate were added and stirred up with the precipitate. After standing several hours (overnight, if convenient) the contents of the tube were stirred again and centrifuged. The clear acetone extract was decanted and the precipitate was stirred up with another portion of acetone and

TABLE III

*Neutral Fat Glycerol Determined in Alcohol-Ether Extracts of Cow Blood Plasma*

Sample No.	Volume of extract	Glycerol added as triolein	Glycerol determined	Glycerol per 100 cc. plasma
	cc.	mg.	mg.	mg.
1	200	0.94	0.84	8.4
	100		1.31	7.4
2	200	0.57	0.42	4.2
	100		0.22	4.4
3	100	0.57	0.75	3.6
	250		0.34	2.7
	200		0.81	2.4
4	100	1.15	1.31	3.2
	200	1.07	0.28	2.8
	200		1.42	3.5
	100		0.12	2.4

recentrifuged. This procedure was repeated until the acetone was colorless in the final washing. Usually four to five washings were required. The washings were added to the original acetone extract. A similar volume of acetone, serving as a blank, was subjected to the subsequent treatment along with the extract.

The acetone was evaporated, boiling tubes being used to prevent bumping, and the residue was extracted several times with petroleum ether. The petroleum ether was evaporated and the residue was saponified and treated as that obtained from the alcohol-ether extracts after precipitation of the phospholipids.

Results of the determination of glycerol in the acetone extracts of jugular vein blood plasma (from a lactating Guernsey cow) are shown in Table IV. Triolein was added to some of the plasma samples before the acetone extraction. A factor of 10, based on a mean molecular weight of 294 for the glyceride fatty acids, was used to convert the value for glycerol to that for neutral fat. The glycerol found averaged 9.0 mg. per 100 cc. of plasma, corresponding to 90 mg. of neutral fat. Glycerol determined similarly in jugular vein blood plasma of a Holstein cow corresponded to 52 mg. of neutral fat per 100 cc.

TABLE IV  
*Neutral Fat Glycerol in Acetone Extracts of Cow Blood Plasma*

Volume of plasma used	Glycerol added as triolein	Glycerol determined		Estimated neu- tral fat per 100 cc. plasma
		Total	Per 100 cc. plasma	
cc.	mg.	mg.	mg.	mg.
15		1.40	9.3	93
15	0.58	1.97	9.3	93
15	0.67	2.03	9.1	91
10		0.89	8.9	89
10	0.46	1.37	9.1	91
10	0.91	1.73	8.2	82

*Arteriovenous Differences in Neutral Fat Glycerol*

Blood from the subcutaneous abdominal vein and internal iliac artery was collected four times from a lactating Ayrshire cow. The cow was of somewhat nervous temperament and had to be hobbled for the collection of the venous blood. Arterial blood was obtained by the rectal approach as described by Graham, Kay, and McIntosh (8). The mammary vein was tapped and arterial blood was taken while the venous blood was being drawn or shortly thereafter. The sampling times, covering the entire period in which both blood samples were obtained, were 7, 12, 6, and 2 minutes, respectively.

The results of the glycerol determinations and neutral fat estimations in these samples are presented in Table V. In three of the samples a noticeable drop in glycerol content between the arterial and venous samples was found. In the last sampling

the glycerol content of the arterial and venous sample was about the same.

To the extent of their reliability, these results indicate the removal of neutral fat by the mammary gland. The conclusiveness of this interpretation is limited, however, in that the one cow employed was not a particularly willing subject and the samples were not obtained under the best conditions. On the other hand, according to Graham *et al.* (8), any disturbance tending to upset the animal during the collection of blood causes the composition of the arterial and venous bloods, representing the metabolism of the mammary gland, to become more nearly alike.

Glycerol was determined in the alcohol-ether extracts of four samplings of arteriovenous blood employed by Maynard *et al.* (1)

TABLE V

*Neutral Fat Glycerol and Estimated Neutral Fat in Simultaneous Samplings of Arterial and Venous Blood Plasma from Lactating Ayrshire Cow*

Date of sampling	Internal iliac artery		Mammary vein		Arteriovenous difference in neutral fat
	Glycerol	Neutral fat	Glycerol	Neutral fat	
<i>July, 1939</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
10	4.1	41	2.7	27	14
12	6.1	61	4.2	42	19
14	6.1	61	3.5	35	26
17	4.5	45	4.4	44	1

in their studies of the blood precursors of milk fat. One of these from a dry cow showed an arteriovenous difference of 3 mg. of neutral fat, whereas three samplings from lactating cows showed arteriovenous differences of 19, 16, and 12 mg. of neutral fat per 100 cc. of plasma based on their glycerol contents.

#### DISCUSSION

In the determination of neutral fat glycerol in blood, by the procedures outlined above, anomalous values appear, at times in the blanks and, presumably, likewise in the unknowns. The blank determinations are readily reproducible when the operations are simple, as with the pure triglycerides, but special precautions are necessary to prevent accumulation of impurities

from materials used during the isolation of the neutral fat glycerol from blood. There is no assurance that the errors in the blanks and in the reagents used for the extractions will be compensatory. Consequently, it is necessary to use purified acetone, ethanol, and petroleum ether in the isolation of the neutral fat glycerol and asbestos instead of filter paper for the final filtration. These precautions make the blank determinations more reproducible and more nearly approach the theoretical values. The variations in the blanks are usually small but in dealing with quantities of glycerol in the range of 0.1 to 0.3 mg. any slight errors in the blanks are magnified considerably in relation to the glycerol being determined.

#### SUMMARY

A method for the determination of 0.1 to 2.5 mg. of glycerol with periodate has been described. The method has been applied to the determination of neutral fat glycerol in bovine blood and its usefulness in the determination of arteriovenous differences in neutral fat has been pointed out. Evidence indicating the removal of neutral fat from blood by the mammary gland has been presented.

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## STUDIES IN BLOOD PRESERVATION\*

### SOME EFFECTS OF CARBON DIOXIDE

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Various changes in shed blood have been reported; only a few will be cited. When blood is exposed to ordinary atmosphere, carbon dioxide is liberated (7) with a resultant increase in pH (6). Another change is in the formation of ammonia. According to Conway (3), ammonia is present only in very small concentrations, if at all, in circulating blood. In shed blood, however, the ammonia concentration rapidly increases, attaining within a few minutes values given as "normal" by most investigators; thereafter, its formation is at a slower rate.

Conway (3) and Conway and Cooke (4) have shown that when blood is shed into an atmosphere of carbon dioxide the concentration of ammonia remains at a low value for some hours.

The purpose of the present investigation is to examine the effect of carbon dioxide in relation to changes in concentrations of ammonia, potassium, and sodium in the plasma of sterile citrated blood occurring over a period of days.

### *Methods*

In each of eight experiments, blood was obtained from a different individual in the usual manner; one-half of the sample was drawn into an atmosphere of carbon dioxide, while the control was collected in air. On the samples so taken, from four to six deter-

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minations of the ammonia content were made at intervals during a 2 week period.

The data of one of these experiments are shown graphically in Fig. 1. In this experiment, ten 50 ml. centrifuge tubes with a diameter of 2.5 cm. were used as containers. Carbon dioxide from a cylinder, filtered through sterile cotton, was introduced into the bottom of five centrifuge tubes so as to displace the air. To each were added 2.5 ml. of 3.5 per cent sodium citrate and 22.5 ml. of blood. For the carbon dioxide experiments, the blood was

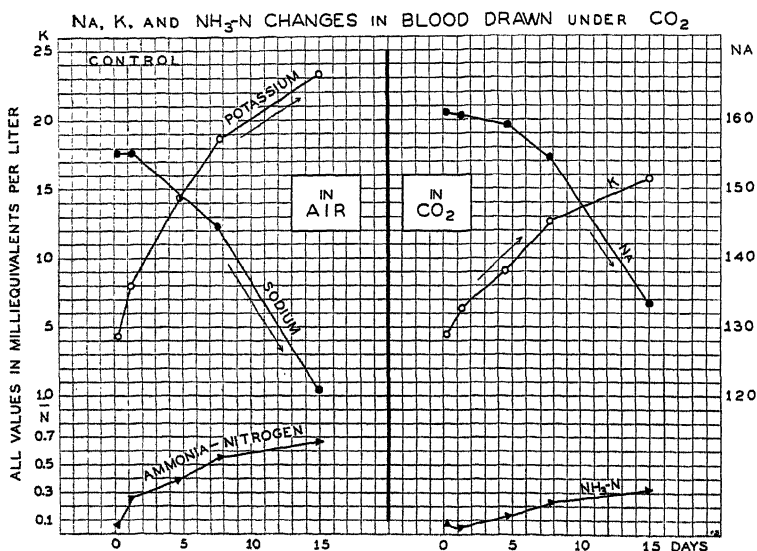


FIG. 1. Concentrations of ammonia nitrogen, potassium, and sodium in plasma of preserved blood, after collection in air (control) and in carbon dioxide. In control, pH 7.76 to 7.69; in CO<sub>2</sub> 7.48 to 7.17.

admitted directly to the bottom of the centrifuge tubes; in the controls, the blood was allowed to flow in at the top. Both sets were closed with sterile rubber stoppers, sealed with paraffin, and placed in a refrigerator at 4°. At intervals during a 2 week period, a carbon dioxide tube and its control were removed from the refrigerator and centrifuged. The supernatant plasma was then drawn off for analyses.

The ammonia was determined by the method of Conway (3), the potassium by a modification (9) of the argenticobaltinitrite

method of Breh and Gaebler (1), and the sodium by the method of Butler and Tuthill (2). Determinations of pH were made on six of the ten samples, with the glass electrode of MacInnes and Longworth (5). Measurements of ammonia, sodium, pH, and the development of color in the potassium determinations were made in a constant temperature room,  $20.5^{\circ} \pm 1^{\circ}$ .

### Results

In each instance the concentration of ammonia in the blood taken in carbon dioxide is less than in the similarly treated control. In both, the concentration of ammonia gradually increases throughout the 2 week period; but at any time, it is consistently less in blood collected in carbon dioxide. Similar conditions are found for potassium and sodium; namely, changes in the concentrations occur during storage, but these are definitely less when the blood is taken in carbon dioxide.

The curves of Fig. 1 are typical in form for the changes observed. At the end of the experiment 27 per cent of intracellular potassium had diffused out in the sample collected in air in contrast to only 16 per cent in the sample collected in carbon dioxide. In a comparison of potassium values, the interfacial area between plasma and cells should be stated (8).

It is evident that the taking of blood directly into an atmosphere of carbon dioxide is effective in retarding changes in the concentrations of plasma ammonia, sodium, and potassium which occur during storage. Such procedure might lengthen the period during which preserved blood could be used for transfusions.

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## BLOOD SUGAR LEVELS IN RATS RECEIVING THE CATARACTOGENIC SUGARS GALACTOSE AND XYLOSE\*

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Numerous investigators (1-5) have confirmed the observation of Mitchell and Dodge (6) and of Mitchell (7) that rats develop cataract when given a diet containing a high percentage of lactose or galactose. Certain other carbohydrates, *viz.* starch, dextrin, maltose, sucrose, fructose, and glucose, do not alter the transparency of the rat lens even when fed as 60 to 70 per cent of the diet (6, 2, 8). The mechanism of this injurious action of galactose has not been satisfactorily explained. The experiments here reported were undertaken to determine whether any of the uninvestigated monosaccharides might share this injurious action with galactose. If so, it should be possible to investigate certain biological properties of these sugars, and, by correlating the results with similar studies on galactose, to arrive at a plausible explanation of the mechanism of this injurious action on rat lenses.

### EXPERIMENTAL

Young rats of both sexes from 20 to 22 days of age and ranging in weight from 30 to 60 gm. were employed. One litter of rats 28 days old and weighing 55 to 67 gm. was used. In all, thirteen litters were employed. Three separate strains were investigated, albinos from Wistar stock, a strain of hooded animals, and a strain of black hybrids.

The rats were kept in individual wire cages with raised screen

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bottoms. Clean distilled water was accessible at all times. One modification of the following basal formula was given to each rat *ad libitum*: casein, commercial edible, 18 per cent; salt mixture, Hubbell, Mendel, and Wakeman (9), 3; cod liver oil,<sup>1</sup> 2; butter fat, 6; yeast, dried,<sup>2</sup> 10; corn-starch, 26; monosaccharide, 35.

One of the following monosaccharides was incorporated in each diet: arabinose, xylose, glucose, galactose, mannose, or fructose. The experiments were controlled so that litter mates received different sugars. The sugars were purchased from the Pfanstiehl Chemical Company. Both technical and c.p. grades of xylose were used. The c.p. grade gave the following analysis:  $[\alpha]_D^{28} = +19.7^\circ$ ; per cent moisture, 0.04; per cent ash, 0.00; reducing ratio, xylose to glucose, 1.01:1. The technical xylose gave the following:  $[\alpha]_D^{28} = +22.9^\circ$ ; per cent moisture, 1.02; per cent ash, 0.019; reducing ratio, xylose to glucose, 0.97:1. Only c.p. arabinose was used. It gave the following analysis:  $[\alpha]_D^{28} = -105.3^\circ$ ; per cent moisture and ash, 0.00; reducing ratio, arabinose to glucose, 0.93:1. c.p. grades of galactose and mannose were employed. The galactose exhibited an  $[\alpha]_D^{28} = +79.5^\circ$  and a reducing ratio, galactose to glucose, of 0.86:1.

Ophthalmoscopic examinations of the eyes of the rats were made daily during the early part of the experimental period and at 3 day intervals thereafter. Before examination the iris was dilated by instillation of a drop of 0.5 per cent atropine sulfate solution into each eye. Occasional observations were made with a slit-lamp. In an attempt to determine whether blood sugar levels could be correlated with the incidence and time of appearance of cataract, blood sugar determinations were made at intervals on 56 of the animals. These determinations were made while the animals had free access to their food. In addition, tolerance curves were run on certain of the animals.

Freely flowing tail blood was used in all determinations. The tail was thoroughly washed with warm water and dried with a clean towel before the sample was taken. Difficulty was experienced in repeatedly obtaining 0.1 cc. of blood from the smaller rats, especially during sugar tolerance tests. To overcome this difficulty the Folin-Malmros method for blood sugar (10) was

<sup>1</sup> Supplied by the E. L. Patch Company, Boston.

<sup>2</sup> Supplied by The Fleischmann Laboratories, New York.

modified slightly to allow use of smaller quantities of blood. The modified technique is as follows:

The usual reagents were employed. Special diluting pipettes calibrated at both 0.025 cc. and 2.5 cc. were made. Blood was drawn to the 0.025 cc. mark, diluted to the 2.5 cc. mark with the dilute tungstic acid mixture, mixed, and then expelled into a clean, dry 15 cc. conical bottom centrifuge tube. After centrifugation, the supernatant liquid was drained into a similar tube and 2 cc. of this filtrate were then measured into a test-tube graduated at 12.5 cc. (Lewis-Benedict tube (11)). 2 cc. of the diluted standard were then placed in a similar tube. To each were added 1 cc. of the 0.4 per cent ferricyanide solution and 0.5 cc. of the carbonate-cyanide solution. Heating and cooling were carried out as in the original method. 2.5 cc. of the colloidal ferric iron solution were added to each tube. Finally, the contents of each tube were diluted to the 12.5 cc. mark and mixed, and the comparison made in a colorimeter. The usual yellow glass filter was employed. Using this technique, we have been successful in carrying out repeated blood sugar determinations on 3 and 4 week-old rats at intervals as frequent as 15 minutes.

### *Results*

The blood sugar levels, expressed as mg. of glucose per 100 cc. of blood, and the incidence and time of appearance of cataract in rats receiving the various diets are given in Tables I and II.

None of the animals receiving diets containing 35 per cent of either glucose, mannose, fructose, or arabinose developed cataracts. In contrast, twenty-nine of the thirty rats placed on a similar diet containing xylose showed definite lenticular opacities. As indicated in Table II, the xylose group showed ophthalmoscopic cataract approximately a day earlier than the litter mate controls receiving galactose. Furthermore, the average time required for the development of mature cataracts in the xylose group was 18.7 days as contrasted with 21.4 days for the galactose group. The incidence of mature cataracts, however, was only about one-half as great in the xylose group as in the galactose group. As seen with the ophthalmoscope the appearance of the cataracts which developed on the two diets was identical. The earliest lens changes appeared at the periphery of the lens, and could be seen only upon *complete* dilatation of the

pupil. Such early changes are easily mistaken for the edge of the iris by one unaccustomed to examining the eye of the rat. Fig. 1 shows the eyes of typical experimental animals.

TABLE I  
*Blood Sugar Levels of Rats on Diets Containing Different Sugars As 35 Per Cent of Diet*

Sugar	No. of rats	No. of determinations	Mean blood sugar as glucose, with probable error
			<i>mg. per 100 cc.</i>
<i>d</i> -Galactose.....	8	26	262 $\pm$ 9.4
<i>d</i> -Xylose.....	21	77	178 $\pm$ 2.3
<i>d</i> -Glucose.....	13	34	132 $\pm$ 1.9
<i>d</i> -Fructose.....	4	14	130 $\pm$ 2.5
<i>d</i> -Mannose.....	4	17	123 $\pm$ 1.3
<i>d</i> -Arabinose.....	6	21	144 $\pm$ 1.9

TABLE II  
*Incidence of Cataract in Rats Receiving Various Sugars As 35 Per Cent of Their Diet*

Sugar	No. of rats	No. showing cataract	Average time of appearance of cataract ophthalmoscopically
			<i>days</i>
<i>d</i> -Galactose.....	11	11	5.8
<i>d</i> -Xylose.....	30	29	4.6
<i>d</i> -Glucose.....	18	0	
<i>d</i> -Fructose.....	4	0	
<i>d</i> -Mannose.....	4	0	
<i>d</i> -Arabinose.....	9	0	

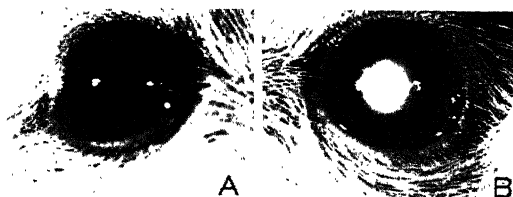


FIG. 1. Enlarged photographs of eyes of typical experimental animals. A, normal eye of rat that received glucose; B, cataract resulting from xylose feeding.

All of the animals receiving xylose developed a frothy diarrhea within a few hours after being placed on the diet. Some abdom-

inal distention was observed. During the 1st week they usually failed to gain in weight. After a variable period of 1 to 3 weeks, however, the distention and diarrhea disappeared and the animals gained at a slightly subnormal rate. None of this group died from the effects of the dietary regimen. The mannose group also developed a mild transitory diarrhea. The average period of observation of this group was 21.3 days, with a maximum of 24 days. A much more severe distention and diarrhea were observed in the animals given arabinose. None of these survived longer than 18 days, the average survival period for nine rats being 10.6 days.

#### DISCUSSION

These results confirm and extend our preliminary experiments (12) on the cataractogenic action of xylose. From these data it is obvious that the statement of Mitchell and Cook (13) that "galactose is unique as an etiologic factor in the production of experimental cataract in rats..." requires some modification. Furthermore, their observation (8), based on a limited number of animals, that "ophthalmoscopic observations on the xylose group revealed some early and rather transitory lens changes which never progressed beyond this stage" seems to indicate that they had observed early cataractous changes in those animals. It is possible that they were dealing with a group of rats particularly resistant to xylose cataract. We observed that rats from the black hybrid strain were more resistant than the others to xylose cataract; none of the four rats of this strain developed mature cataract and one showed such slight lenticular changes that we have not classed them as cataractous. Another animal of this strain showed an apparent regression of early cataractous changes.

Table I shows that significantly higher blood sugar levels were found in those rats receiving the cataractogenic sugars, xylose and galactose. The average for the galactose group is lower than that for a similar series previously reported from this laboratory (2) for rats given a diet containing 60 per cent galactose but no corn-starch. Cori and Cori (14) demonstrated that the blood sugar level in rats is definitely lower following the administration of a mixture of galactose and glucose than it is after administration of galactose alone. Since the diet used in the present

experiments contained 26 per cent corn-starch, which gives rise to glucose on digestion, it seems reasonable to assume that the difference we observed is partly due to the same effect as reported by Cori and Cori.

The average blood sugar level of 77 determinations on twenty-one rats given xylose is considerably above that of eight determinations on two rats reported by Mitchell and coworkers (8). Donhoffer's observation (15) that different strains of rats show varying rates of absorption of certain sugars may have some bearing on this discrepancy between our results and the results of Mitchell and coworkers. From the work of Miller and Lewis (16) and of Blatherwick *et al.* (17) it seems reasonable to assume that at least a part of the increase in the blood sugar level in the rats given the xylose diet was due to the presence of xylose in the blood. Of the monosaccharides which did not produce lenticular opacities when administered to the extent of 35 per cent of the diet, arabinose was the only one which raised the blood sugar level appreciably, and this not nearly so much as did xylose.

Thus, the ability of the monosaccharides to raise the blood sugar level of the rat seems to accompany the cataractogenic property of the sugars investigated. However, there appears to be no direct relationship between the time required for the development of cataract in rats and the hyperglycemic effect of the sugars tested. Hence, it would appear that the hyperglycemic effect of a given monosaccharide may not be the only factor determining whether that particular sugar is cataractogenic for rats. In this regard, it is interesting to note that both xylose and galactose may be considered, according to Rosanoff's classification of monosaccharides (18), as derived from *d*-threose. Similarly, all of the sugars which proved to be non-injurious to the lens under the conditions employed in these experiments are derivatives of *d*-erythrose. An investigation of other biological properties of these sugars and of other monosaccharides and their derivatives, and a correlation of properties with chemical configuration, may assist in the solution of this problem.

#### SUMMARY

A modification of the Folin-Malmros blood sugar method is described which permits determination of blood sugar on 0.025 cc. samples of blood.

Data are presented which show that when glucose, fructose, mannose, or arabinose constituted 35 per cent of the diet given young rats the animals did not develop cataracts. Similar diets containing xylose or galactose were cataractogenic. Animals receiving the latter carbohydrates showed elevated blood sugar levels, but the degree of hyperglycemia bore no simple relationship to the rapidity of cataract formation.

It is concluded that galactose is not unique in its cataractogenic activity, but that this property is shared by at least one other monosaccharide, xylose. The configurational relationships of the sugars investigated are pointed out.

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## OSMOTIC PRESSURE, MOLECULAR WEIGHT, AND DISSOCIATION OF LIMULUS HEMOCYANIN

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*Limulus* hemocyanin, the copper-containing, respiratory blood protein of the horseshoe crab, has been found to comprise four components in aqueous isoelectric solution (17, 31, 33). The molecular weight of the largest of these is 2,040,000 (34) or greater (31), but that of the smallest is known less precisely. Thus Cohn (11) from ultrafiltration experiments in 1925 found in solutions of *Limulus* hemocyanin a protein of molecular weight intermediate between that of hemoglobin and pseudoglobulin, which, by recent estimation, have weights of 68,000 (1, 32) and 178,000 (8); and Redfield, Coolidge, and Shotts (27) from a consideration of the copper content of the protein and other data estimated the probable molecular weight to be 73,000.

Burk and Greenberg (10, 18) and others (30, 35, 36) have shown that urea dissociated many proteins. A more exact evaluation of the size of the smaller protein component may therefore be possible through molecular weight estimation in urea solution. Thus certain proteins which are split by urea (horse hemoglobin, myogen, amandin, and excelsin) have been found to dissociate into units of molecular weight in the neighborhood of 35,000.

The close relationships recently established between the hemocyanins and the oxidases (tyrosinase (16) and polyphenol oxidase (22, 24)) make the dissociation of copper-containing proteins one of significance for enzyme reactions. Thus crude polyphenol oxidase extract loses its activity in concentrated urea solution, whereas pepsin, an enzymic protein which is not dissociated by urea, retains full activity (30).

\* National Research Council Fellow in Biochemistry, 1929-31, during which time most of the experiments reported in this paper were carried out.

The rôle in dissociation of the copper of *Limulus* hemocyanin, which apparently is linked directly to amino acids (14, 15), is also considered in the present report of molecular weight estimations from osmotic pressure measurements upon *Limulus* hemocyanin in aqueous and aqueous urea solutions.

#### EXPERIMENTAL

The oxyhemocyanin used in the osmotic experiments was obtained from the serum<sup>1</sup> of *Limulus polyphemus* and purified by precipitation and dialysis according to the method of Redfield, Coolidge, and Shotts (27). The osmotic pressure measurements were carried out at 0° by the method used in previous work (9, 6, 10, 18). The hemocyanin was allowed to remain in the urea solutions at least 10 days before the measurements were completed.

*Molecular Weight in Urea Solution*—Measurements of osmotic pressure with respect to increasing concentration of hemocyanin were carried out in 6.66 M urea at a reaction near the isoelectric point. The isoelectric point of the protein in 6.66 M urea containing 0.05 M buffer, determined osmotically, was found to be at about pH 6.2 (Fig. 1).<sup>2</sup> The results are given in Table I, where it will be seen that the osmotic pressure is not proportional to the concentration but increases more rapidly. The solutions were therefore not ideal.  $P/C$  plotted against  $C$  gave points through which a straight line may be drawn (Fig. 2) and hence, as previously shown (6), the data may be corrected for deviations from the ideal solution law by means of a corrected concentration, determined by the equation,

$$C_0 = \frac{PC}{100 - BC^2} \quad (1)$$

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<sup>1</sup> I am indebted to Professor A. C. Redfield for a supply of *Limulus* serum. This was preserved with half saturated ammonium sulfate.

<sup>2</sup> Osmotic measurements in a different series at pH 6.1 and 6.3 showed that the  $P/C$  values at these two hydrogen ion concentrations were the same within experimental error. These measurements do not appear in the series in Fig. 1 because of difference in  $C$ . The shape of the curve, however, was determined in part from these data.

where  $C_o$  = corrected concentration of the protein and  $B$  = a constant, equal to the slope of the line obtained by plotting  $P/C$  against  $C$ .

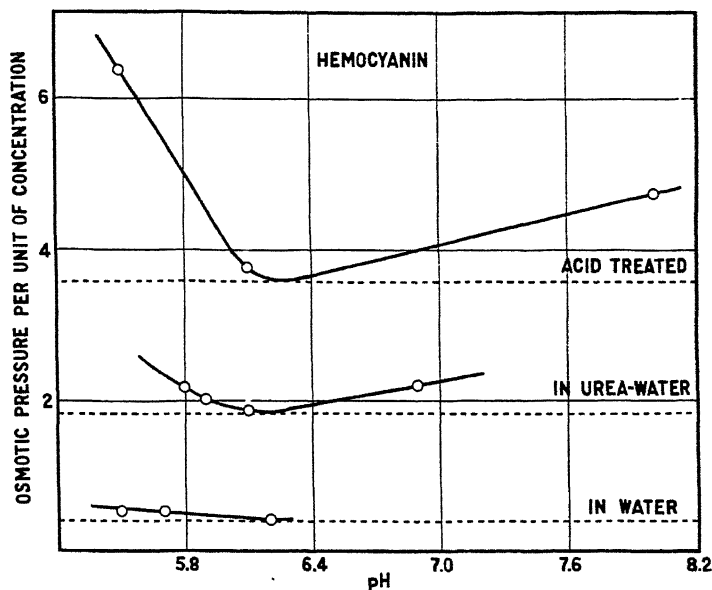


FIG. 1. The stepwise dissociation of *Limulus* hemocyanin. The influence of the hydrogen ion activity on the osmotic pressure. The dotted lines represent the minimum osmotic pressure, corresponding to that of the protein without the presence of a Donnan membrane equilibrium. Lower curve, hemocyanin in 0.05 M phosphate buffer; protein concentration, 7.62 to 7.84 gm. per 100 cc. of solvent. Middle curve, hemocyanin in 6.66 M urea, 0.05 M with respect to acetate or phosphate buffer; protein concentration, 1.70 to 1.71 gm. per 100 cc. Upper curve, acid-treated hemocyanin in 6.66 M urea, 0.05 M with respect to acetate or phosphate buffer; protein concentration, 1.02 to 1.20 gm. per 100 cc.

The mean molecular weight of *Limulus* hemocyanin in urea solution would appear to be close to 142,000 (Table I), as calculated from the van't Hoff-Morse equation,

$$M = RT \frac{C_o}{P} \quad (2)$$

where  $M$  = molecular weight in gm. of dry protein,  $C_0$  = concentration in gm. per 100 cc. of solvent,  $P$  = osmotic pressure in cm. of  $H_2O$  of density 1, and  $RT$  = gas constant  $\times$  absolute temperature =  $2.315 \times 10^5$  (100 cc.  $\times$  cm. of  $H_2O$  per gm. molecule) at

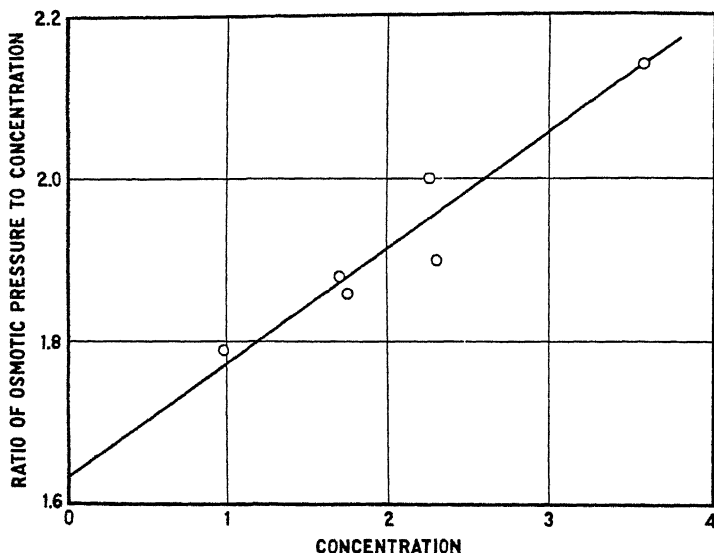


FIG. 2. Relation between the osmotic pressure per unit of concentration and the concentration of *Limulus* hemocyanin. Solvent, 6.66 M urea, 0.05 M phosphate buffer; pH about 6.2. The concentration is expressed in gm. per 100 cc. of solvent and the osmotic pressure in cm. of water.

0°. This value falls within the limits of molecular weight to be expected from the early ultrafiltration experiments (11).<sup>3</sup>

<sup>3</sup> A rough estimate of the molecular weight of the smallest *Limulus* hemocyanin component observed in the ultracentrifuge may be made from its reported sedimentation constant,  $s_{20} = 5.9 \times 10^{-13}$  (31), and an assumed dissymmetry constant. The dissymmetry constants,  $f/f_0$ , of five other dissociation components from different hemocyanins have been determined by Svedberg (see (31) Table I), and these range from 1.4 to 1.9. If the mean value 1.6 is assumed for the *Limulus* hemocyanin dissociation component, its molecular weight is 150,000 as calculated from the equation  $M = 5230$

$$\left( s_{20} \frac{f}{f_0} \right)^{\frac{2}{3}}.$$

*Molecular Weight in Aqueous Salt Solution*—Two measurements upon hemocyanin in aqueous salt solution at the isoelectric point, pH 6.2 to 6.4 (28), were carried out, one in 0.2 M and the other in

TABLE I  
*Molecular Weight of Limulus Hemocyanin from Osmotic Pressure Measurements in Urea Solution*

Solvent, 6.66 M urea, 0.05 M in phosphate buffer; pH about 6.2; temperature, 0°.

Experiment No.	C, concentration per 100 cc. solvent	P, osmotic pressure	g,* osmotic coefficient	C <sub>o</sub> † corrected concentration	$\frac{P}{C_o}$	M, mol. wt.
	gm.	cm. H <sub>2</sub> O				gm.
1	0.98	1.75	1.08	1.06	1.65	140,000
3	1.70	3.20	1.15	1.95	1.64	141,000
4	1.75	3.26	1.15	2.01	1.62	143,000
5	2.26	4.52	1.19	2.68	1.68	138,000
6	2.30	4.38	1.20	2.77	1.58	147,000
7	3.58	7.67	1.30	4.67	1.64	141,000
Mean.....						142,000

\* Calculated from the equation,  $g = C_o/C$ .

† Calculated from the equation,  $C_o = PC/PC - BC^2$ , where, from Fig. 2,  $B = 0.142$ .

TABLE II  
*Molecular Weight of Limulus Hemocyanin from Osmotic Pressure Measurements in Aqueous Salt Solution*

Solvent, phosphate buffer solution; temperature, 0°.

Experiment No.	pH	Concentration of buffer	P, osmotic pressure	C, protein concentration per 100 cc. solvent	$\frac{P}{C}$	M, mol. wt. (uncorrected)
		mole per l.	cm. H <sub>2</sub> O	gm.		gm.
512	6.2	0.2	3.07	7.65	0.40	579,000
509	6.2	0.5	3.53	8.34	0.42	551,000
Mean.....						565,000

0.5 M phosphate buffer. The results, given in Table II, show that the variation of  $P/C$  with salt concentration is small. The average of the measurements yields a value of 565,000 for the mean

molecular weight of the hemocyanin from the horseshoe crab. Comparison of this value with that obtained for urea solutions confirms the ultracentrifugal observations (31) that *Limulus* hemocyanin dissociates.<sup>4</sup>

*Molecular Weight of Acid-Treated Hemocyanin in Urea Solution*—It is well known that the copper in hemocyanin becomes detached in moderately acid solution (2-5, 20, 23, 26). A few experiments were carried out upon acid-treated hemocyanin in order to determine whether copper removal affects the molecular weight. Acid-treated hemocyanin was prepared as follows: Thrice precipitated hemocyanin was dissolved in 100 cc. of 5 per cent ammonium sulfate and poured into 10 liters of distilled water. 1 M HCl was added slowly until the pH of the solution was slightly below 3, and then the solution was neutralized after a few minutes. The precipitated protein was washed repeatedly (by decantation) with 0.1 N KCl and subsequently with distilled water.

The results of osmotic measurements upon this protein material in 6.66 M urea at various pH values are shown plotted in Fig. 1. The osmotic pressure at the minimum in the curve in Fig. 1 is 3.6 cm. of water per unit of protein concentration, or approximately twice as great as that of hemocyanin not treated by acid and similarly measured. The molecular weight of acid-treated denatured *Limulus* hemocyanin, calculated from this pressure, assuming its deviation from the ideal solution law to be the same as for normal hemocyanin in urea (osmotic coefficient,

<sup>4</sup> In addition to dissociating *Limulus* hemocyanin, urea also decolorizes the protein and renders it insoluble in isoelectric salt solution. Experiments upon *Cancer* hemocyanin showed that —SH groups were liberated in the copper-free protein (24) in the presence of urea, but were not detectable in urea solutions of normal *Cancer* hemocyanin. Thus when *Cancer* hemocyanin (or crude polyphenol oxidase extract—potato peel juice) was added to urea solutions of edestin, the —SH groups present (7, 19) were destroyed (negative nitroprusside test). When guaiacum (21) was added to urea solutions of hemocyanin (or of crude polyphenol oxidase), it was converted into guaiacum blue. Since the guaiacum reaction is a well known oxidation reaction, and since hemocyanin in the presence of urea behaves like an oxidase (or a methemocyanin (*cf.* (13))), it appears that normal hemocyanin oxidizes its own —SH groups in urea solution. This effect of urea in conferring oxidative properties upon hemocyanin was not observed in the case of any non-metallic protein.

1.08 at  $C = 1.02$ ), is 69,000, a value of the order of one-half<sup>5</sup> that for urea-treated *Limulus* hemocyanin.<sup>6</sup>

*Molecular Weight from Chemical Analysis*—An estimation of the minimal molecular weight of *Limulus* hemocyanin has been made by applying the usual procedure (12) to its copper, sulfur, and amino acid content. The mean value obtained is 36,700 (Table III). This estimate is based purely on stoichiometrical relationships, and is approximately one-quarter of the minimal value obtained from osmotic pressure measurements.

TABLE III

*Minimal Molecular Weight of Limulus Hemocyanin from Chemical Analysis*

Protein constituent	Weight of constituent present	Weight containing 1 atom or molecule	Assumed No. of atoms or molecules	Minimal mol. wt.
	<i>per cent</i>	<i>gm.</i>		<i>gm.</i>
Cystine.....	1.94 (25)	12,383	3	37,100
Methionine.....	2.76 (25)	5,404	7	37,800
Tryptophane...	4.52 (29)	4,516	8	36,100
Tyrosine.....	4.50 (29)	4,024	9	36,200
Histidine.....	4.52 (25)	3,431	11	37,800
Arginine.....	6.37 (25)	2,733	13	35,500
Lysine.....	8.92 (25)	1,638	22	36,000
Sulfur.....	1.22 (25)	2,623	14	36,700
Copper.....	0.173 (27)	36,745	1	36,700
Mean.....				36,700

The figures in parentheses are bibliographic reference numbers.

For convenience the hemocyanin unit of molecular weight 147,000 (see osmotic pressure) will be referred to in this work as

<sup>5</sup> No reduction in the molecular weight of *Cancer* hemocyanin occurred when copper was removed from the native protein. This was shown by comparative osmotic pressure measurements upon normal and upon copper-free hemocyanin prepared by the cyanide procedure of Kubowitz (24). Both proteins were dissociated by urea. In native hemocyanin (*Cancer*) the copper therefore appears combined in such a way that a breakage of its linkages is unaccompanied by protein dissociation, whereas in denatured (acid-treated) hemocyanin (*Limulus*) dissociation appears to be coincident with rupture of copper bonds.

<sup>6</sup> Chemical tests showed that urea-treated *Limulus* hemocyanin contained copper.



the fundamental<sup>7</sup> hemocyanin unit, since smaller copper-containing units have not yet been reported.

*Relation between Fundamental Hemocyanin Unit and Prosthetic Group*—The number of prosthetic groups (14, 15) attached to the fundamental hemocyanin unit may be ascertained by comparing the number of copper atoms in the complex and in this unit, since according to Conant and his coworkers all the copper in *Limulus* hemocyanin appears to be in the prosthetic group (14, 15).

The analytical data of Conant, Dersch, and Mydans (14), given in Table IV, suggest 4 atoms of copper per mole of amino acid prosthetic group. This is supported by the data of Mazur (25), whose reported value of 0.08 per cent "sulfate" S in *Limulus*

TABLE IV  
*Minimal Molecular Weight of Prosthetic Groups of Limulus Hemocyanin from Chemical Analysis*

Constituent	Percentage of constituent	Weight containing 1 atom	Assumed No. of atoms	Mol. wt.
		gm.		gm.
Amino N.....	0.58	2415	1	2415
Cu.....	14.2	448	4	1792
S.....	6.3	508	4	2032
Mean.....				2080

hemocyanin agrees fairly well with that calculated on the assumption that there are 4 atoms of Cu and 4 atoms of S<sup>8</sup> in the prosthetic group; namely,  $(4 \times 32 \times 100)/147,000 = 0.087$ . Since 4 atoms of Cu also appear to be present in the fundamental hemocyanin unit (Table III), it would follow that this unit contains 1 prosthetic group.

The fundamental hemocyanin unit and the prosthetic group do not appear to be linked together by means of copper bonds, since

<sup>7</sup> Fundamental with respect to size, but not necessarily with respect to chemical composition.

<sup>8</sup> Present as "sulfate" S, since the prosthetic group contains neither methionine nor cystine S, whereas *Limulus* hemocyanin contains methionine, cystine, and "sulfate" S (cf. (25)).

removal of metal by dialysis against cyanide does not remove the prosthetic group, for resynthesis of pigment takes place on addition of copper salt to the dialyzed solution (24).

The writer is indebted to Professor E. J. Cohn, and also to Professor R. M. Ferry, Professor J. T. Edsall, and Dr. J. P. Greenstein for reading the manuscript of this paper and making helpful suggestions.

#### SUMMARY

1. From osmotic pressure measurements on *Limulus* hemocyanin in isoelectric urea solutions, the mean molecular weight (corrected for deviations) was found to be 142,000, or smaller than that observed in aqueous isoelectric buffer solutions.

2. *Limulus* hemocyanin, after treatment with acid at about pH 3, which removes the copper from the protein, was found to have a molecular weight in urea solution of 69,000, or approximately one-half that of copper-containing hemocyanin in urea.

3. The minimal molecular weight of *Limulus* hemocyanin estimated from chemical analysis appears to be 36,800.

4. The *Limulus* hemocyanin unit of molecular weight 147,000 appears to possess one prosthetic group containing 4 atoms of copper.

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## ANALYSES OF URINARY PROTEIN AND VARIOUS FRACTIONS OF HUMAN AND PIG SERUM PROTEIN

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Interest in the chemical composition and identification of urinary protein has been stimulated by the work of Block *et al.* (1) who suggest that urinary protein is chemically identical with total serum protein and is changed to an albumin in the urine by the physical environment. For the proof of such a concept it is essential to have exact and complete amino acid analyses of urinary and serum proteins. One of the purposes of this paper is to furnish such analyses.

Further interest is aroused by Block's (2) separation of serum protein by various salting-out procedures into many protein fractions with widely different molecular ratios of the amino acids, arginine to lysine (10:9 to 10:92). This variation in ratio suggests the possibility of the formation of artificial products from one large aggregate by the reagents employed rather than a separation of the serum proteins into known entities of albumins and globulins.

### EXPERIMENTAL

The serum was separated from 1500 ml. of blood obtained from three healthy young men and combined for analysis. 200 ml. of serum were coagulated by heat at pH 5, filtered, and the precipitate washed with hot water until chloride- and sulfate-free.

\* The data in this paper have been taken from a thesis presented by William A. Murrill to the Horace H. Rackham School of Graduate Studies of the University of Michigan in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

It was then washed several times with 95 per cent alcohol, once with absolute alcohol, and several times with ether and dried in a vacuum desiccator over sulfuric acid for several days. This was considered to be total normal serum protein. The remaining 430 ml. of serum were fractionated quantitatively into albumin and globulin by the procedure of Howe (3) and washed and dried as before. In addition, a 500 ml. sample of blood was obtained from a nephritic patient who had a daily output of 7 gm. of urinary protein. The serum was separated and total serum protein was prepared as above.

Urinary protein (Sample A) was collected from this same patient over a period of several weeks, while subsisting on a diet free of egg protein. The intake was 100 gm. of protein and 3000 calories per day. A second sample of urinary protein (Sample B) was obtained over a period of 2 weeks while this individual was fed a diet in which the protein (35 gm.) was supplied essentially by eggs and which yielded 1500 calories per day. In both cases the collection of urinary protein was started after the subject had been on the diet for 1 week.

The albumin to globulin ratios of the serum and urinary proteins were obtained by the method of Howe (3). Moisture and ash were determined by the usual procedure for the urinary protein samples. The values for serum protein were corrected for ash and moisture by the following procedure. Assuming 16 per cent as the average nitrogen content for serum proteins corrected for moisture and ash, we multiplied our various analytical values by the ratio of 16 over the experimentally determined nitrogen content. The total nitrogen was obtained by the micro-Kjeldahl method of Pregl and amide nitrogen by the aeration method (4). Total sulfur was determined by the Parr bomb method. Cystine was determined by the Sullivan method, as modified by Rossouw and Wilken-Jorden (5), tyrosine and tryptophane by the methods of Folin and Marenzi (6), and basic amino acids by the methods of Block (7). The histidine was isolated by use of nitranilic acid (7).

#### DISCUSSION

The total nitrogen content of the various fractions of normal serum protein varied from 13.48 to 14.72 per cent and was in

general of the same order of magnitude as that of nephritic total serum protein (Table I). The variation probably was due to a difference in the moisture content, as shown in the case of the two samples of urinary protein, the values being 16.26 and 16.16 per cent respectively when corrected for ash (Sample A, 0.23 per cent; Sample B, 0.09 per cent) and moisture (Sample A, 8.8 per cent, and Sample B, 4.9 per cent). A significant difference was found in the amount of cystine, tyrosine, tryptophane, lysine, and ar-

TABLE I

*Analyses of Human Serum and Urinary Proteins*

All values except the total nitrogen are corrected for ash and moisture as described in the text.

Protein	Albumin Globulin	Total N	Sulfur	Cystine	Sulfur accounted for by cystine	Tyrosine	Tryptophane	Histidine	Arginine	Lysine
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Total serum, normal....	2.0	14.28	1.62	4.32	71	5.56	1.65	2.47	5.20	9.63
“ “ nephritic..	2.6	14.72	1.62	4.24	70	5.45	1.58	2.53	5.22	9.64
Albumin, normal.....		13.48		4.75		4.94	0.82	2.58	5.30	10.40
Globulin, “ .....		14.00		3.43		6.38	2.42	2.34	4.70	7.00
Theoretical values*....				4.70		5.0	0.88	2.57	5.28	10.27
Urine protein, Sample A†	24	14.80	1.84	5.7	82.4	5.1	0.90	2.46	5.70	10.46
“ “ “ B.	24	15.33	1.87	6.0	86.1	5.2	0.92	2.58	5.50	10.35

\* Values calculated for a serum protein with an albumin to globulin ratio of 24:1, our values for albumin and globulin being used. See the text.

† Samples A and B were collected while the subject was on different diets. See the text for the details.

ginine in serum globulin as compared with serum albumin (Table I).

The values for sulfur and various amino acids in total normal serum protein are in close agreement with those obtained for total nephritic serum protein (Table I). These findings are in agreement with those of Metzger (8) and Tuchman and Reiner (9). However, in view of the work of Lang and Braun (10), Schenck and Kunstmann (11), Alving and Mirsky (12), and Dirr (13)

we might have expected differences, because these workers present evidence to show that serum proteins are not of constant composition.

The percentages of sulfur and of all the amino acids determined except histidine are significantly different in albumin and globulin. In our preparation the cystine content of human serum albumin was 4.75 per cent as compared to 3.43 per cent for the globulin. Other workers (8, 14-17) find similar differences in the cystine content of serum albumin and globulin obtained from different species (human, horse, goose, and ox). Hewitt (18) separated serum albumin into three fractions and obtained 5.8 per cent cystine as the highest value, in the fraction referred to as crystalalbumin. The lowest cystine content of an albumin fraction (1.8 per cent) was found in seroglycoid. Abderhalden and Siebel (19) obtained for horse serum albumin 4.5 per cent tyrosine and 0.83 per cent tryptophane, and for globulin 5.73 per cent tyrosine and 2.12 per cent tryptophane. These are in agreement with our values for human serum albumin (tyrosine, 4.94 per cent; tryptophane, 0.82) and globulin (tyrosine, 6.38 per cent; tryptophane, 2.42). The histidine values obtained by us are higher than those previously reported (1, 20) for serum proteins. This can be explained by the fact that we used the nitranilic acid method of Block (7), which gives higher values. No differences between the histidine content of human serum albumin and human serum globulin were observed. This is contrary to the findings of Block (20) who made similar studies with cattle serum. The arginine and lysine values for albumin are 5.3 and 10.4 per cent respectively as contrasted to 4.7 and 7.0 per cent for the globulin. These absolute values are higher but the relative values are similar to those for cattle serum (20).

In order to ascertain whether urinary protein is total serum protein (1, 21) or mostly albumin (22), comparison of the sulfur and amino acid contents of the urinary protein, total serum protein, albumin, and globulin is significant (Table I). The cystine values for urinary protein (5.7 and 6.0 per cent) are higher than those for our unpurified sample of serum albumin but agree with the value of 5.8 per cent for crystalalbumin (23) and with the figure of 6.07 per cent obtained for human serum albumin by Tuchman and Reiner (9). If the values obtained for tyrosine,

tryptophane, arginine, and lysine of human serum albumin are compared with the urinary protein values (Table I), one observes very close agreement. The albumin to globulin ratio for urinary protein was found to be 24:1 (Table I). A close agreement exists between the amino acid composition of the urinary protein as determined and that which would be anticipated if the urinary protein were a mixture of serum proteins in the above ratio (designated as "Theoretical values" in Table I) or entirely serum albumin. The evidence here presented suggests that serum protein is composed of at least two distinctly different protein com-

TABLE II  
*Analyses of Pig Serum Protein Fractions*

All values except the total nitrogen are corrected for ash and moisture as described in the text.

Fraction	Nitrogen			Cys- tine	Tyro- sine	Tryp- to- phane	His- tidine	Argi- nine	Lysine
	Total	Amide of total	Hu- min of total						
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Saturated MgSO <sub>4</sub> fil- trate, 25°.....	14.01	6.81	1.76	5.39	5.20	1.08	2.9	5.8	9.5
30% Na <sub>2</sub> SO <sub>4</sub> ppt.....	14.35	8.35	3.00	2.84	5.92	1.23	*	5.7	8.4
20% MgSO <sub>4</sub> " .....	13.35	9.46	1.60	3.08	6.04	2.13	2.0	5.3	6.9
Saturated MgSO <sub>4</sub> ppt., 37°.....	15.22	8.20	1.83	2.75	5.90	2.16	2.0	5.4	6.5
15% Na <sub>2</sub> SO <sub>4</sub> ppt.....	15.08	8.03	1.60	2.76	6.10	2.13	1.8	5.3	6.2

\* Determination lost.

ponents, since the albumin in the native state must have different properties from the other constituents to be selectively excreted through the kidney.

Serological comparisons also indicate to us that urinary protein is not total serum protein but a fraction thereof.<sup>1</sup>

Our findings that urinary protein is not identical with total serum protein in so far as amino acid content is concerned does not corroborate the work of Block *et al.* (1) who found that urinary protein is the same as total serum protein. In view of this dis-

<sup>1</sup> Murrill, W. A., Soule, M. H., and Newburgh, L. H., unpublished data.



crepancy and since Block used this finding as evidence that serum protein fractions as ordinarily prepared are artifacts rather than real entities, we fractionated fresh pig serum by the salting-out procedures of Block (2) and determined on each fraction nitrogen, sulfur, and six amino acids (Table II). These data indicate no great variation in the amino acid values. The differences are of the same order of magnitude that exist between the contents of the amino acids found for human serum albumin and globulin (Table I). The greatest variations of fractions of pig serum were in the cystine (2.75 to 5.39 per cent), tryptophane (1.08 to 2.16 per cent), and lysine (6.2 to 9.5 per cent) contents. The arginine to lysine ratios varied from 10:14 to 10:20 as contrasted to 10:9 to 10:92 reported by Block for cattle serum (2). The data afford no evidence that the experimental procedure of salting-out effects any significant fractionation other than the usual separation into the components commonly designated as albumin and globulin.

#### SUMMARY

1. A comparative study of the composition of serum albumin, serum globulin, and urinary protein has been made by determining the sulfur, nitrogen, and various amino acids. The results indicate that urinary protein is either all serum albumin or is a mixture correctly represented by the albumin to globulin ratio as found in the urine.

2. Two samples of urinary protein collected while the subject subsisted on different dietary régimes show identical analyses.

3. Pig serum was fractionated by the procedures of Block and the various fractions were analyzed. No evidence was obtained to indicate that the various fractions differed widely in composition, notably in their lysine content, as reported by Block for the protein fractions of beef serum.

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## ASSAYS OF URINE FROM RHESUS MONKEYS FOR PREGNANEDIOL AND OTHER STEROIDS\*

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The discovery of the relationship of progesterone to the pregnanediols (1) has proved important both theoretically and clinically. Since it has been shown that the titer of pregnanediol, present in human urine as sodium pregnanediol glucuronide, reflects the rate of secretion of progesterone and hence the activity of the corpus luteum, it is possible roughly to determine the time of ovulation in the menstrual cycle (2-5). Since in the pregnant woman the pregnanediol level rises gradually in normal cases according to a certain broad curve, it is possible in pathological cases, as for example in habitual abortion, to diagnose the character of an existing hormonal deficiency (6-10).

Inasmuch as the rhesus monkey has come to be the standard primate animal for the study of menstrual phenomena and of pregnancy, it seemed of interest to determine whether this species metabolizes progesterone as does man.

Before we proceed to the discussion of the joint studies made by the present authors, it is necessary to report some previous assays of pregnancy urine made on monkeys of the Carnegie colony. These results are here reported for the first time with the permission of the experimenters, Dr. E. B. Astwood and Dr. Paul G. Weil.

In 1937 Astwood made assays on the pooled urines from the pregnant monkeys listed in Table I and also the urines of six

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non-pregnant females and one male. In no case was pregnanediol glucuronidate recoverable even in traces. It was also found that none of the 50 mg. of free pregnanediol injected subcutaneously into a dog or 90 mg. injected into a monkey was recoverable in the urine, whereas much of the 110 mg. of the water-soluble glucuronidate injected into an adult rabbit rapidly reached the urine.

In 1938 Weil (11), applying his method of extracting free pregnanediol where present, again failed to find any trace of this compound in the urine of several pregnant monkeys of the Carnegie colony.

TABLE I  
*Collection of Monkey Urine for Analysis by Dr. Astwood*

Carnegie accession No.	Weight	Date of conception	Date of parturition	Urine collection
	<i>kg.</i>		<i>1937</i>	
281	7	Jan. 16, 1937	July 11	Mar. 9-29
391	6	Nov. 28, 1936	May 12	" 9-29; Apr. 1-18
469	7	Feb. 1, 1937	June 23	" 9-29; " 1-18
H20	Large		Mar. 27	" 9-29; " 1-18
H22	"		May 3	" 9-29; " 1-18
T18	"		Apr. 18	Apr. 3-20

Further evidence of species differences in the metabolism of progesterone is afforded by certain studies which it is pertinent to cite here. Westphal and Buxton (12) were unable to recover pregnanediol glucuronidate from pregnant rabbits and cats, although this substance is present in the urines of other species (pregnant cow (13), pregnant mare (14), bull (15)). The allo-pregnanediols are characteristic of the latter (13-16), while pregnanediol-3( $\alpha$ ),20( $\alpha$ ) is characteristic of human pregnancy urine (1), which yields some allo-pregnanediols also (17, 18). Comparing man and monkey with regard to the fate of injected progesterone, we note that this compound administered to either women (19, 20) or men (21) is excreted by the kidneys as sodium pregnanediol glucuronidate. This is not true of the monkey (12).

In view, therefore, of this fundamental difference between man and monkey in the metabolism of progesterone it seemed desir-

able to pursue the subject further. It was decided, first, to make a more complete assay of pregnancy urine of the monkey for various steroidal compounds and, second, to attempt to recover end-products of theelin or of progesterone administered in large amounts to monkeys.

*Assays of Urine from Untreated Pregnant Rhesus Monkeys*

In the present study 12 gallons of urine were collected from three pregnant monkeys whose protocols read as follows:

*Monkey 609*—This 4.5 kilo female conceived in consequence of a single coitus on December 23, 1938. Urine was collected from January 30 until March 6, 1939, when the animal was used for another purpose.

*Monkey 622*—Conception occurred in the case of this animal (weight 4.5 kilos) on December 6, 1938. Urine was collected from January 30 to March 20, 1939. On May 24 the monkey delivered a 430 gm. female baby, which it nursed.

*Monkey 636*—On arrival from India on April 16, 1938, this female was in possession of three new born babies, having probably kidnaped two of the three from cage mates. The monkey weighed 4780 gm. when acquired by the Carnegie colony 2 weeks later. On December 5 the monkey conceived again. Urine was collected from January 30 to March 20, 1939. On April 27, the 145th day of gestation, a two-headed monster was born dead.

*Examination of Pregnancy Urines*

12 gallons of urine of the three pregnant rhesus monkeys listed above were hydrolyzed by heating at 95° for 75 minutes with 10 per cent hydrochloric acid. The product was well extracted with butanol and the solvent removed by vacuum distillation. The residue was hydrolyzed by steam distillation with sodium hydroxide for 2 hours. The total product was extracted with a large volume of ether, which was washed with water and evaporated to a syrup.

*Carbinol Fraction*—The neutral portion from the alkaline hydrolysis was dried by distillation of benzene from it. To the residue were added 8 gm. of succinic anhydride and 10 cc. of pyridine. This was heated for 1 hour on a steam bath, dissolved in ether, and the pyridine removed by hydrochloric acid. The

succinates were removed by shaking with potassium carbonate solution. These were hydrolyzed by refluxing with alcoholic potassium hydroxide for 1 hour. The carbinol fraction was extracted and gave 2.9 gm. of material.

This was dissolved in 25 cc. of ethyl alcohol and a solution of 1 gm. of digitonin in 50 cc. of alcohol added. After standing overnight, the precipitate was filtered and dried; weight 390 mg. This was decomposed by heating with pyridine, and the product crystallized from alcohol. It gave a product melting at 146–147° which gave no depression in melting point when mixed with cholesterol; weight 35 mg.

The filtrate from the cholesterol was dissolved in alcohol and treated with bromine until a faint color remained. This was then treated with alcoholic digitonin, but only a faint precipitate was obtained, showing that no saturated  $\beta$ -sterols were present. Further proof of this was afforded by debromination and oxidation at 20° with chromic acid in acetic acid. No allopregnanedione was obtained, which is very insoluble and readily isolated. There was also no equistanol present, which has been found in all urines examined except human urine.

The mother liquors from the first digitonin precipitation were evaporated to dryness and taken up in ether and filtered. The residue was treated with Girard's reagent in alcohol, but no appreciable amount of ketones was present. The non-ketonic carbinol fraction (weight 2.4 gm.) was sublimed in a high vacuum. The first fraction taken up to 100° was an oil weighing 300 mg. This upon oxidation at 20° gave only acids and was probably aliphatic carbinols, as it had quite a fruity odor. The next fraction distilling at from 100–125° weighed 320 mg. This also gave only acids upon oxidation at 20°. The next fraction taken from 125–225° over a 40 hour period weighed 950 mg. It was very soluble in ether, benzene, acetone, and ethanol, from which the pregnanediols readily crystallize if present. It resisted crystallization even from dilute acetone, coming out as an oil. The total product was added to 25 cc. of xylene and refluxed with sodium for 9 hours. If allopregnanediol were present, it would have been converted into the  $\beta$  form at C-3 which precipitates with digitonin. Upon treatment with digitonin in alcohol there was only a small precipitation, showing the absence of allopregnanediol. The

product was then oxidized with chromic acid in acetic acid at 20°, yielding 310 mg. of ketones after treatment with Girard's reagent. These would not crystallize from any of the solvents from which allopregnanedione crystallizes. Their semicarbazones were very soluble in alcohol, which is in contrast to the almost completely insoluble semicarbazone of allopregnanedione.

The non-distillable fraction of carbinols which consisted of a dark resin gave only a gelatinous precipitate in small amount upon attempted crystallization. This was oxidized by chromic anhydride in acetic acid and the ketonic fraction removed by Girard's reagent consisted of 240 mg. of product. This was sublimed in a high vacuum and the portion distilling at 100–150° crystallized from dilute acetone and methyl alcohol. After three such crystallizations and one from pentane 4 mg. of product were obtained, melting at 126–129°, which gave no depression in melting point when mixed with androstanedione.

*Analysis*— $C_{19}H_{28}O_2$ . Calculated, C 79.1, H 9.8; found, C 78.7, H 9.6

*Hydrocarbon Fraction*—The ether extract of the succinates was distilled in a high vacuum at 90–120°. This fraction gave upon crystallization from acetone a product melting at 63–64°; yield 35 mg. It gave no depression in melting point when mixed with the urinary hydrocarbon obtained from human pregnancy urine and mare's pregnancy urine.

*Analysis*— $C_{28}H_{48}$ . Calculated, C 85.3, H 14.8; found, C 85.2, H 14.6

*Phenolic Fraction*—The alkaline extract of the original hydrolysis was acidified and extracted with ether. This was washed with sodium carbonate solution to free it of acids. It was then dissolved in sodium hydroxide and extracted with ether. The alkaline layer was acidified and extracted with ether. The phenolic fraction consisting of 2.6 gm. was distilled in a high vacuum. Much low boiling material distilled below 120° which was very fluid and smelled strongly of cresols. The fraction distilling from 120–240° weighed 1.1 gm. and was dissolved in alcohol and heated with semicarbazide hydrochloride and sodium acetate for 1 hour, the alcohol being distilled off. The residue was extracted with ether, heated with water, and filtered. It gave 8 mg. of crude product which upon recrystallization from alcohol



gave 2 to 3 mg. of a product which melted at 245–248° and gave no depression in melting point when mixed with estrone semicarbazone. The amount was too small for further characterization.

*Urine of Pregnant Monkey Injected with Estrone*

*Administration of Estrone*—Monkey 661 weighed 5330 gm. when received on November 17, 1938; 6580 gm. a year later. On February 8, 1939, the monkey conceived. On April 28, the 78th day of gestation, hypodermic injections of theelin were begun. 1.125 gm. of crystalline theelin were dissolved in 20 cc. of Mazola oil (maize oil). The container was warmed and shaken each day before removal of a portion for injection. 1 cc. containing 56.25 mg. of theelin was administered daily except that on Saturdays a double dose was given to make up for omission of injections on Sundays. However, on the last day only 0.5 cc. was left; a total of 1.097 gm. of theelin therefore was injected in the 20 day period.

The injected hormone had certain physiological effects. The sex skin of the animal was, of course, greatly congested, that is, brilliantly red; but the edema of the sex skin was much less marked than is usual with such large doses in non-pregnant animals. The only swellings were loose folds in the epipubic and perineal regions. On June 5, the 116th day of gestation, the female gave birth to a somewhat dehydrated fetus which had a crown-rump length normally attained by the monkey fetus at 100 days of development. The sex color did not diminish in intensity after the abortion, as is usually the case; in fact continued unabated into July, when observations were discontinued. The long persistence of the estrogenic effect was probably due to slow absorption of the highly concentrated oil solution.

It is worthy of note that after the April molt the animal failed to acquire a new coat of hair until well into the fall.

Urine was collected from the animal in three lots of 2 gallons each. Roughly, the first batch covered the period of actual injections, the second, the succeeding 3 weeks, and the third, a period following the abortion.

*Assay of Urine*—The urine was worked up in 2 gallon lots as collected. Each lot was hydrolyzed by heating at 95° for 1 hour with 10 per cent hydrochloric acid. It was thoroughly extracted with butanol and the solvent removed *in vacuo*. The residue was

dissolved in alcohol, ether added, and the alcohol removed by washing with water. A tar precipitated and was removed from the ether by filtering through norit. The filtrate was shaken with aqueous sodium hydroxide to remove acids and phenols. The aqueous layer was acidified, extracted with ether, and the ethereal solution washed with sodium bicarbonate solution and then water. The ether was evaporated, giving a residue of 0.3 gm. of phenolic material. This was dissolved in ethyl alcohol and heated for 2 hours with 100 mg. of semicarbazide hydrochloride and 110 mg. of sodium acetate, the alcohol being distilled in the meantime. Ether was added and the product filtered and washed well with ether. It was then washed with water and a small amount of cold alcohol followed by ether. This gave 7 mg. of product melting at 249–253° with decomposition. The second 2 gallons of urine yielded 13 mg. of product, whereas the third 2 gallons of urine collected after abortion gave 28 mg. of product. The semicarbazones were mixed and recrystallized from alcohol to give a product melting at 252–254° with decomposition. It gave no depression in melting point when mixed with estrone semicarbazone.

Upon hydrolysis with dilute alcohol sulfuric acid and crystallization from 50 per cent alcohol, the product yielded estrone, m.p. 258–259°, which gave no depression with an authentic sample.

*Analysis*— $C_{18}H_{22}O_2$ . Calculated, C 79.9, H 8.3; found, C 79.8, H 8.2

No additional phenolic fraction was obtained from the neutral fraction after alkaline hydrolysis of the total combined extracts of the 6 gallons of urine. When worked up as described in the first case, the product did not contain a trace of any of the three pregnanediols common to other pregnancy urines. A small quantity of cholesterol was isolated.

#### *Urine of Pregnant Monkey Receiving Progesterone*

*Administration of Hormone*—Monkey 657 was received into the colony on November 17, 1938, weighing 5330 gm. The animal ovulated and conceived on February 8, 1939. Beginning on April 28, the 79th day of gestation, it received subcutaneously daily injections of 53.5 mg. of progesterone in 1 cc. of oil, except

on three Saturdays when it received 107 mg. and three Sundays when none was given; on the last day (May 17) only 36 mg. were left for injection. The animal therefore received a total of 1.040 gm. in 20 days. About 2 gallons of urine were collected in 33 days, as against almost double that quantity furnished by Monkey 661 receiving theelin. The female showed no unfavorable symptoms at any time except a slight blanching of the sex skin (to 6 on a scale of 10) for several weeks after the injections were stopped. There was no blanching during or immediately after the period of injection. Such a failure to react promptly to progesterone is attributable to the pregnancy, since in a castrate receiving estrone the reddened sex skin promptly blanches upon injection of progesterone.

On July 25, the 167th day of gestation, Monkey 652 gave birth to a 460 gm. male baby which it nursed until November 14, when the infant was needed for neurological studies; 167 days constitute almost the exact average of the gestation period for the rhesus monkey.

It is thus seen that this monkey metabolized a relatively huge quantity of progesterone in mid-pregnancy without untoward effect either on mother or infant.

*Assay of Urine*—The urine was collected for 33 days and amounted to approximately 2 gallons. This was processed as described in the first case. It did not yield a trace of any of the three pregnanediols present in other pregnancy urines. There was not enough estrone present for isolation. The only product obtained from the carbinol fraction was cholesterol. Even on oxidation of the carbinol fraction, isolation of the ketones by means of Girard's reagent, and conversion to the semicarbazones produced no insoluble semicarbazones which are characteristic of the semicarbazones of both pregnanediol and allopregnanediol.

A control experiment on the technique of isolation of the above products was run on a 2 gallon sample of human pregnancy urine, from which the pregnanediols were isolated in the expected quantities.

#### SUMMARY

The steroidal content of the urine of the pregnant rhesus monkey was investigated and it was found that it does not contain even a

trace of the pregnanediols common to other pregnancy urines. A pregnant monkey was injected with over 1 gm. of estrone in 20 days and upon examination the urine was found to contain only a small portion of the total estrone injected. The estrone caused the death of the fetus. Injection of progesterone (over 1 gm. in 20 days) into the pregnant monkey failed to produce even a trace of the pregnanediols in its urine. The progesterone had no unfavorable effect on either the mother or fetus.

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# A METHOD FOR ACETONE BODIES IN BLOOD, APPLICABLE TO THE DETERMINATION OF SMALL AMOUNTS OF MERCURY

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3 years ago the author faced the need of a blood acetone body method that would be sufficiently delicate for use in determining the low values present during the onset of ketosis, sufficiently accurate for use with arteriovenous differences, and technically simple enough to allow a number of simultaneous determinations without inconvenience. The method described below is believed to meet these requirements and has proved itself satisfactory in a number of studies on acetone body metabolism. It depends upon the determination of mercury in the Denigès's precipitate formed essentially according to the technique of Van Slyke (1). The suppression of ferric thiocyanate color in the presence of minimal amounts of thiocyanate and an excess of ferric nitrate is proportional to the amount of mercuric ion present, and permits determination of the mercury in the precipitate formed from the filtrate representing 1 ml. of normal blood.

## *Reagents—*

0.002 N mercuric nitrate standard solution. Dissolve 0.2166 gm. of red  $\text{HgO}$  in about 100 ml. of distilled water to which 5 ml. of concentrated nitric acid have been added, and make up to 1 liter with distilled water.

Modified Denigès reagent. Dissolve 70 gm. of c.p.  $\text{HgSO}_4$  in 6.0 N  $\text{H}_2\text{SO}_4$  and make up to 1 liter with 6.0 N  $\text{H}_2\text{SO}_4$ . Merck's brand of mercuric sulfate has been satisfactory, which makes it unnecessary to prepare this solution by dissolving red mercuric oxide in sulfuric acid as recommended by Van Slyke.

Thiocyanate solution (approximately 0.01 N). Dissolve about 900 mg. of NaCNS or 1.1 gm. of KCNS in 1 liter of distilled water. Since standard curves should be obtained for known amounts of mercuric ion each time new solutions are made, it is not necessary to standardize the thiocyanate solution. It should, however, be adjusted so that when 1 ml. is added to 50 ml. of a solution containing 5 ml. of concentrated nitric acid and 2 ml. of 50 per cent ferric nitrate solution the color that appears gives a reading of between 25 and 30 on the photometer scale, or a convenient reading on any other type of photoelectric colorimeter. A thiocyanate solution of half the above strength is preferable for the acetone body values found in normal blood. The thiocyanate solution may be protected against mold formation by the addition of 2 gm. of sodium benzoate per liter; this has no effect on color development or intensity.

50 per cent ferric nitrate solution. 100 gm. of c.p.  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  are dissolved in water and made up to 200 ml. with the addition of 2 ml. of concentrated nitric acid.

5 per cent potassium dichromate in distilled water.

### *Procedure*

In brief, protein-free blood filtrate is refluxed with the Denigès reagent according to Van Slyke (1), the precipitate is retained on a sintered glass filter, after careful washing the precipitate is dissolved by boiling concentrated nitric acid, an excess of ferric nitrate and a small amount of thiocyanate are added to the dissolved precipitate, and the intensity of the color is determined in the photoelectric colorimeter.

The protein-free filtrate is best prepared by use of the second zinc sulfate technique of Somogyi (2), in which the blood is laked by an acidified solution of zinc sulfate and precipitation accomplished by the addition of sodium hydroxide solution. The mercuric sulfate precipitation as described by Barnes (3) may also be used but is less convenient. Values obtained on the two types of filtrate from the same sample of blood have checked within the limits of error of the method.

The removal of glucose is necessary only when the blood sugar level is above 300 mg. per 100 ml. If hyperglycemia of this degree is present, the sugar should be removed by treating 10

ml. of the filtrate with 4 ml. of 10 per cent copper sulfate, adding sufficient solid calcium hydroxide to make the mixture alkaline, allowing it to stand for 30 minutes, and filtering. Allowance must of course be made for the dilution when the acetone body content of blood so treated is calculated.

Into a 125 ml., Pyrex round bottom flask with 24/40 standard taper joint are placed 10 ml. of filtrate and 4 ml. of modified Denigès reagent. A pin-head-sized particle of alundum that has been cleaned by boiling with concentrated nitric acid and has then been thoroughly washed with distilled water is placed in the flask. It is then connected with a Liebig type of Pyrex condenser without a drip tip and with a 400 mm. water jacket, the condenser terminating in a 24/40 male joint. The flask rests upon a sand bath in a pan 13 cm. in diameter and is further protected from the direct heat of the flame by an asbestos sheet 25 cm. square and with a central opening 6 cm. in diameter that is placed beneath the sand bath. These precautions are essential to obtain slow and even boiling and to prevent overheating of any solution that spatters on the sides of the flask. The sand bath is heated by a microburner, with the flame just high enough to produce even boiling. If any bumping should occur, a second alundum particle must be added through the condenser tube; the alundum should be a moderately coarse grade. After boiling has been steady for a few minutes, 1.0 ml. of 5 per cent potassium dichromate is added through the condenser tube and the contents of the flask are allowed to reflux for  $1\frac{1}{2}$  hours.

When the flask is cool enough to be held in the hand the contents are filtered with suction through a No. 3G4 (finest obtainable) Jena fritted glass filter funnel of 30 ml. capacity that is fitted to a 1 liter suction flask. The flask is rinsed three times with 15 to 20 ml. quantities of distilled water and the rinsings poured through the funnel. The sides of the funnel should be washed down with the last rinsing. The precipitate on the filter funnel is then further washed twice with distilled water. If the precipitate shows a tendency to creep up the sides, the washing should be continued until it is confined to the lower third of the funnel. Care must be taken that the alundum particle has been washed out on the filter, since some Denigès's precipitate will be adherent to it. The suction is then stopped, the filter



funnel removed, and a  $20 \times 200$  mm. test-tube graduated at 50 ml. is placed in the suction flask. The filter funnel is replaced so that its neck extends into this test-tube. Then 5 ml. of concentrated nitric acid are brought to a boil in a 50 ml. beaker, and when just boiling are poured into the funnel. The funnel is rotated to insure contact of the hot acid with all parts where precipitate may be lodged, and gentle suction is applied to draw the acid through the funnel in about 1 minute. The funnel is washed with sufficient distilled water to bring the total volume in the test-tube to about 45 ml. The test-tube is removed and 2 ml. of 50 per cent ferric nitrate solution are added to its contents which are then brought to the 50 ml. mark with distilled water. At this point the tube may be stoppered and the remainder of the procedure deferred if desired. Mercuric ion is determined in the contents of the tube by adding 1 ml. of thiocyanate solution, mixing, and reading in the photoelectric colorimeter (the Cenco Sheard-Sanford photometer with a blue filter has been used). The photometer reading is converted into mg. of mercury by reading from a standard curve.

The standard reference curve is prepared by placing varying amounts (from 0.5 to 5 ml.) of 0.002 N  $\text{Hg}(\text{NO}_3)_2$  solution in  $20 \times 200$  mm. test-tubes graduated at 50 ml., adding 5 ml. of concentrated nitric acid and 2 ml. of 50 per cent ferric nitrate to each, making up to the mark with distilled water, adding 1 ml. of thiocyanate solution, mixing, and reading in the photometer. Each ml. of 0.002 N  $\text{Hg}(\text{NO}_3)_2$  solution contains 2 mg. of mercury. The standard curve so obtained is a straight line when plotted on semilogarithmic paper, showing that color depression is in direct proportion to the amount of mercury present.

The flasks are best cleaned between determinations by rinsing with concentrated HCl, which is the best solvent for traces of mercury compounds that may remain.

The upper limit of the method as outlined above depends upon the exact concentration of the thiocyanate solution, which for convenience is left somewhat variable. In general if the blood contains more than 15 to 18 mg. of acetone bodies (as  $\beta$ -hydroxybutyric acid) per 100 ml., the amount of mercury in the precipitate from 10 ml. of blood filtrate will either decolorize the solution completely or will produce such a faint color that it

cannot be read with accuracy. Such determinations need not be lost. If the solution is colorless or judged to be too faint for accuracy, a 2nd ml. of thiocyanate may be added; if this does not produce color, a 3rd and if necessary a 4th ml. of thiocyanate may be used. 4 ml. of thiocyanate reagent will carry the range slightly above 60 mg. of acetone bodies per 100 ml. of blood, a value which has seldom been exceeded in the author's experiments to date. It is suggested that if more than this amount of acetone bodies, expressed as  $\beta$ -hydroxybutyric acid, is present the blood filtrate be diluted. Above this range the volume of precipitate is such that solution in 5 ml. of boiling nitric acid is slow and a faint yellow color is imparted by the amount of dichromate in the precipitate. Standard curves must of course be set up for the values between 15 and 60 mg. per 100 ml. if they are to be encountered.

The readings obtained in terms of mg. of mercury are converted into acetone bodies expressed as mg. of  $\beta$ -hydroxybutyric acid per 100 ml. of blood by multiplying by 15.4, or as mg. of acetone by multiplying by 6.33. The author has preferred to express his determinations in terms of  $\beta$ -hydroxybutyric acid, since this appears to be that member of the group present in largest amount in ketosis.

Acetone plus diacetic acid is determined alone by refluxing for 40 minutes without the addition of dichromate, filtering, and proceeding as above. If  $\beta$ -hydroxybutyric acid is to be determined separately, 2 ml. of 6.0 N  $\text{H}_2\text{SO}_4$  are added to 10 ml. of filtrate and the mixture boiled 10 minutes to drive off acetone and diacetic acid. This is made up to volume with distilled water, 2 ml. of a solution containing 140 gm. of  $\text{HgSO}_4$  per liter of 6.0 N  $\text{H}_2\text{SO}_4$  added, and the remainder of the procedure carried out as above.

#### EXPERIMENTAL

During the preparation of this article the contribution of Barnes and Wick (4) on the same subject was published. The method presented by these authors appears to be a considerable improvement over those previously available but is believed to be no more accurate and perhaps somewhat more laborious than that here described. Since Barnes and Wick have carefully reviewed the

literature and discussed the indications for an improved method, only the directly pertinent literature will be noted in the present communication.

The concentration of dichromate has been increased above that originally recommended by Van Slyke (1) to avoid the necessity of removing glucose from every sample of filtrate. The increase in dichromate does not, as is shown in Table I, affect the recovery of  $\beta$ -hydroxybutyric acid in the absence of glucose. It does prevent glucose from depressing recovery up to concentrations

TABLE I

*Effect of Varying Glucose Concentrations on Recovery of  $\beta$ -Hydroxybutyric Acid from 10 Ml. of Pure Solution*

Glucose added	0.5 ml. 5 per cent dichromate added		Glucose added	1.0 ml. 5 per cent dichromate added	
	$\beta$ -Hydroxybutyric acid added	Per cent recovery		$\beta$ -Hydroxybutyric acid added	Per cent recovery
mg.	mg.		mg.	mg.	
0	0.083	101	0	0.083	98
0	0.083	99	0	0.083	99
0	0.086	97	0	0.086	104
0	0.086	104	0	0.086	103
1.0	0.089	90	1.0	0.083	98
2.0	0.089	88	2.0	0.083	94
3.0	0.089	81	3.0	0.083	96
4.0	0.089	78	3.0	0.089	98
			4.0	0.083	84
			4.0	0.086	90
			4.0	0.086	85

of this sugar corresponding to 300 mg. per cent in blood (3 mg. per 10 ml. of filtrate). The addition of 0.5 ml. of 5 per cent dichromate to the reflux flask gives the concentration recommended by Van Slyke, which as shown in Table I is insufficient to abolish the glucose effect even at normal blood sugar levels. Decreased recovery in the presence of glucose appears to be due to reduction of the dichromate before complete oxidation of the  $\beta$ -hydroxybutyric acid has occurred. Too great an increase in dichromate leads to more rapid  $\beta$ -hydroxybutyric acid oxidation at low glucose levels and a decrease in the amount of precipitate

formed. The use of 1 ml. of 5 per cent dichromate at blood sugar levels below 300 mg. per cent and removal of glucose at higher levels removes interference by this sugar and simplifies the procedure.

Blank determinations on distilled water should show no precipitate greater than the error of the method, which for low concentrations of acetone bodies is about 0.1 mg. per 100 ml. of blood. The mean of ten blank runs is 0.0037 mg. of mercury, standard deviation 0.002; this is equivalent to 0.06 mg. of  $\beta$ -hydroxybutyric acid per 100 ml. of blood.

The mercury equivalent of  $\beta$ -hydroxybutyric acid was determined on samples of the calcium zinc salt prepared by the author according to the method of Shaffer and Marriott (5). One lot of sodium salt from which the calcium zinc salt was made was prepared by the author by reduction of ethyl acetoacetate with sodium amalgam; another lot of sodium salt was made available by the courtesy of Dr. D. L. Tabern. Dr. T. E. Friedemann kindly sent the author several gm. of calcium zinc salt which was also used to check the mercury equivalent. These three preparations of calcium zinc salt all had the same mercury equivalent within the limits of experimental error. The mean of twenty determinations of mercury equivalent that were made in Chicago is 6.36 with a standard error of 0.08. The mean of twenty-five determinations made in Memphis is 6.62, standard error 0.047. The mean of all determinations is 6.45, standard error 0.067. The author has for convenience used 6.5 as the amount of mercury present in the precipitate from 1 part of  $\beta$ -hydroxybutyric acid (mercury equivalent), since this is a more convenient figure than the actual mean and one which is equally probable according to statistical analysis of the data. On the basis of Van Slyke's data (1) the mercury equivalent of  $\beta$ -hydroxybutyric acid can be calculated as 6.49.

The mean mercury equivalent of acetone was found to be 14.8, standard error 0.5, in eight determinations. The equivalent of acetone as calculated from Van Slyke's data (1) is 15.4. A convenient figure for use is 15.0, since this is statistically as probable as 14.8.

In Table II are presented data from experiments in which  $\beta$ -hydroxybutyric acid was added to whole blood as a solution

of the calcium zinc salt, and the amount recoverable in the protein-free filtrate determined. The proteins were precipitated either by mercuric sulfate or zinc hydroxide. The recovery is satisfactory in both types of filtrate.

TABLE II

*Recovery of  $\beta$ -Hydroxybutyric Acid from Blood after Addition of Known Amounts in the Form of the Calcium Zinc Salt*

Sample No.	Added	Found	Recovered	Per cent recovery	Remarks
	<i>mg. per ml.</i>	<i>mg. per ml.</i>	<i>mg. per ml.</i>		
1	0	0.0115	0.0103		Proteins pptd. by mercuric sulfate
	0	0.0092			
	0.0817	0.0936		101.9	
	0.0817	0.0898		97.4	
	0.0817	0.0881		95.1	
	0.0817	0.0936		101.9	
2	0	0.0053	0.0075		Proteins pptd. by mercuric sulfate
	0	0.0086			
	0	0.0086			
	0.0817	0.0857		95.7	
	0.0817	0.0869		99.3	
	0.0817	0.0844		94.1	
3	0	0.0098	0.0092		Proteins pptd. by zinc hydroxide
	0	0.0086			
	0.0475	0.0565		99.5	
	0.0475	0.0550		96.4	
	0.0794	0.0865		97.9	
	0.0794	0.0890		100.8	
4	0	0.0064	0.0070		Proteins pptd. by zinc hydroxide
	0	0.0076			
	0.0408	0.0471		98.5	
	0.0817	0.0862		96.8	

Barnes and Wick (4) have suggested that the composition of the Denigès's precipitate may vary when the concentration of acetone bodies is low, although they present no evidence for this view. The tables of Van Slyke (1) show no such variation and the concentrations of acetone used by him covered a wide range,

the lowest being but twice as high as that commonly present in the present method when applied to normal blood. However, this question has been reinvestigated by determining the recovery of  $\beta$ -hydroxybutyric acid from pure solutions at various concentrations; the results are given in Table III. It will be noted that at the lowest concentrations (0.01 and 0.015 mg., which correspond to 1.0 and 1.5 mg. per 100 ml. of blood) the recovery is actually somewhat low, ranging from 85 to 92 per cent. With

TABLE III  
*Recovery of  $\beta$ -Hydroxybutyric Acid at Various Concentrations*  
(Pure Solutions)

Added	Hg found	$\beta$ -Hydroxybutyric acid found	Per cent recovery
mg.	mg.	mg.	
0.010	0.055	0.0085	85
0.010	0.060	0.0092	92
0.015	0.089	0.0137	91
0.015	0.086	0.0133	89
0.020	0.128	0.0197	99
0.020	0.135	0.0208	104
0.030	0.187	0.0288	96
0.030	0.190	0.0292	97
0.040	0.264	0.0406	102
0.040	0.245	0.0378	95
0.050	0.326	0.0502	100
0.050	0.332	0.0511	102
0.100	0.665	0.1022	102
0.100	0.654	0.1007	101
0.100	0.620	0.0955	96
0.100	0.630	0.0970	97
0.200	1.25	0.193	97
0.200	1.24	0.191	96

higher concentrations, up to 0.20 mg. corresponding to 20 mg. per cent in blood, the recovery is within 5 per cent of the expected value. It is possible that the somewhat low recoveries at small concentrations are due to changes in the composition of the precipitate; it is equally possible that they are due to a slight degree of solubility of the precipitate, and in this case the error would also appear in the method of Barnes and Wick. These authors do not present data on recovery at such low concentrations.

Other factors that may affect the accuracy of the method are the conditions under which the solution is refluxed and the presence of interfering ions. It is necessary to emphasize the need for smooth boiling and avoidance of excessive heat on the sides of the flask. Small amounts of chloride or oxalate ions will increase the intensity of the color. These will be absent if all glassware is kept scrupulously clean. The sulfate ion is a constituent of the Denigès's precipitate, there being a ratio of 1:0.18 between mercury and sulfate. Sulfate added in excess of this proportion to the solutions used in obtaining the standard curve does not change the photometer reading and it is therefore concluded that the amount of sulfate ion liberated when the precipitate is dissolved will not be an interfering factor. Traces of zinc, lead, copper, barium, ammonium, or phosphate ions do not affect the color.

The rate of fading increases with increasing concentration of nitric acid. It has therefore been necessary to limit the amount of nitric acid used to that which is adequate to dissolve the precipitate. Under the conditions given and with fresh reagents a solution containing no mercury faded in 30 minutes by an amount corresponding to 0.14 mg. of  $\beta$ -hydroxybutyric acid per 100 ml. of blood. A solution containing 0.6 mg. of mercury faded inappreciably in 30 minutes. The rate of fading appears to increase in some cases with increasing age of the reagents and should therefore be checked occasionally.

It is obvious that the photometric determination of color depression by the mercuric ion is applicable to the determination of mercury from any source if the mercury can be obtained in solution substantially free of interfering ions. No specific recommendations need be made, since each source of mercury will present its own problems, but it may be pointed out that when the mercury can be split off by refluxing with boiling nitric acid, the mercury content of organic compounds may be readily determined by this method.

In Table IV are presented values for blood acetone bodies found at various periods after the last meal in dog and man. The non-fasting values are much lower than those reported by Van Slyke and Fitz (6) who found 2.2 to 4.6 mg. per cent expressed as  $\beta$ -hydroxybutyric acid. Barnes and Wick (4) report 0.5 to 1.0 mg. per cent as acetone or about twice these figures as  $\beta$ -hy-

droxybutyric acid. Hubbard (7), whose method while laborious appears capable of considerable accuracy, gives data which when recalculated in terms of  $\beta$ -hydroxybutyric acid yield values between 0.4 and 2.0 mg. per cent. The present method then gives data for normal blood of dog and man that compare favorably with those of Hubbard and are slightly lower than those of Barnes and Wick, since the author's range for many determinations

TABLE IV

*Acetone Bodies (Expressed As  $\beta$ -Hydroxybutyric Acid) in Blood of Man and Dog in Non-Fasting and Fasting States*

The values for dogs were determined from arterial blood samples, for man from venous samples.

	Hrs. after last meal	Total acetone bodies	Acetone + diacetic acid
		mg. per cent	mg. per cent
Dog 1	48	0.8	
	96	1.0	
	192	3.1	
" 2	48	2.2	
	96	4.5	
	192	5.8	
" 3	18	1.1	
" 4	18	1.3	
	72	2.4	
	120	4.0	
" 5	18	0.7	
Man 1	16	0.8	
	40	24.8	7.1
	64	60.5	23.8
" 2	16	0.6	
	40	2.5	0.7
	64	36.8	12.6

is from 0.4 to 1.5 mg. per cent. It is interesting to note that the acetone bodies reach much higher values during fasting in the human than in the dog, which correlates with the lesser ketonuria of the latter species.

## SUMMARY

A method is described for acetone bodies in blood which depends upon the formation of the Denigès's precipitate under conditions essentially those used by Van Slyke, isolation of the precipitate,



and determination of the mercury content of the precipitate by means of the specific interference of mercury with the color formed when thiocyanate is added to an excess of ferric nitrate. It is believed that the method may be useful in the determination of mercury in other materials when interfering ions (especially chloride, sulfate, and oxalate) can be excluded. As described, the method is believed to be accurate within  $\pm 5$  per cent for the concentration of acetone bodies encountered in ketosis and within somewhat larger limits for the amounts present in normal blood.

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## CHLOROPHYLLASE

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Chlorophyllase is a relatively specific esterase concerned with the phytol ester linkage in chlorophyll. It is unusual because it operates in high concentrations of alcohol and acetone, and it is but slightly affected by many common inhibitors, but it can be inactivated by heat, and the reaction has been reversed, so that its enzymatic nature will be assumed here. With chlorophyll as substrate, there will be formed either the acid chlorophyllide or its simple alkyl derivative, depending upon the solvent employed.

Chlorophyll *a*,  $C_{32}H_{30}N_4MgO(COOCH_3)(COOC_{20}H_{39})$ ,<sup>1</sup> with chlorophyllase yields (1) in EtOH, 25°, ethyl chlorophyllide *a*, (*COOEt*), and phytol,  $C_{20}H_{39}OH$ ; (2) in acetone, 25°, chlorophyllide *a*, (*COOH*), and phytol; (3) in water, 75°, chlorophyllide *a* and phytol.

Ethyl chlorophyllides *a* and *b* are the "crystalline chlorophylls" first observed by Borodin (1) and explained in terms of chlorophyllase by Willstätter and Stoll (2). The hydrolysis in hot water was discovered by B. E. Lesley and J. W. Shumate.<sup>2</sup>

Mayer (3) has made several quantitative studies on chlorophyll hydrolysis in acetone. With a purified enzyme meal, optimal conditions were pH 6.2, a 66 per cent acetone concentration, and a temperature of 25°. The same optimal temperature was found (2) for ethanol, at an 80 per cent concentration. We are in general agreement with the above findings, which need little elaboration. With an active enzyme, the acetone concentration may be varied from 40 to 70 per cent with substantially equal rates of hydrolysis, but the alcohol optimum has narrower limits,

<sup>1</sup> Only the italicized portion is repeated. The remainder is unchanged.

<sup>2</sup> Of the California Packing Corporation.

about 70 to 80 per cent being most effective. The water system for the fresh material has a definite optimum at 75°. It will be observed later that the rate at 50° is of comparable magnitude provided the leaf has previously been frozen. This indicates that accessibility of substrate to enzyme plays an important part in determining the extent of hydrolysis.

Mayer successfully hydrolyzed chlorophyll, allomerized chlorophyll, and pheophytin in acetone. He found chlorophyll *a* to be hydrolyzed faster than chlorophyll *b* by a factor of 1.8. By washing the leaf powder with water, the activity was lost, but it could be restored by addition of electrolytes. KCN had little effect on activity. Mayer also found great variation in the quantity of enzyme in various plants throughout the growing season.

The purpose of this paper is to show that with different plants radically different results are obtained with these solvents. Thus chlorophyllase from figwort is highly active in 80 per cent ethanol, but inactive in hot water; from spinach, on the other hand, it is highly active at certain seasons in hot water, but very weak in ethanol. A considerable number of plants have been examined, of which the following have been studied in some detail: sunflower, *Helianthus annuus*; spinach, *Spinacia oleracea*; figwort, *Scrophularia californica*; cow-parsnip, *Heracleum lanatum*; wild oat, *Avena fatua*.

*Enzyme Sources*—The plant tissue (fresh, frozen, or dried and powdered) was used directly for the greater part of the work. Extracts with water, buffer mixtures, dilute alcohol, and acetone were inactive. Powdered leaf meal after extraction with 80 per cent acetone usually retained 20 to 50 per cent of the activity of the original and was used for some studies.

Highly active alcohol systems in plants are readily detected by formation of characteristic crystals of methyl or ethyl chlorophyllide. A leaf section about 1 cm.  $\times$  0.5 cm. is cut into narrow strips 0.5 mm. wide and suspended in ethanol between a slide and cover-glass. It is allowed to stand in an atmosphere of alcohol (a Petri dish serves the purpose) for 24 hours, then dried at room temperature, and examined under the microscope. *Scrophularia* showed highest activity with typical greenish black triangular and hexagonal crystals. Spinach did not yield crystals probably because of the weakness of the system and possibly in

part because of other changes, oxidative in character. It was, however, possible to obtain crystals from spinach by addition of *Scrophularia* enzyme.

The limitations of the crystal method are twofold. It cannot be conveniently made quantitative, and where the enzyme is weak, results are negative.

Quantitative methods were devised, based on the insolubility of the chlorophyllide in petroleum ether. In the first method, 80 per cent acetone leaf extracts were prepared, and an aliquot fractionated with petroleum ether and this solvent. By spectrophotometric measurement of the original, in acetone, and the portion which could not be transferred to petroleum ether, the percentage hydrolyzed could be calculated. Measurements were made at 6650, 6450, 6200, and 5950 Å., corresponding roughly to the maxima for components *a* and *b* in aqueous acetone.

It was of some interest that the values at 6450 Å. were consistently too low. This is because the absorption maximum for the phytol-free derivative is 30 to 50 Å. further toward the red than the maximum for chlorophyll. Consequently, on the steep portion of the curve (6500 to 6300 Å.), there is a very significant change, and calculations based on 6450 Å. are therefore omitted from the average.

In the second method, the pigments are partitioned between petroleum ether and 80 per cent acetone. The latter is transferred to diethyl ether. Both fractions are dried with anhydrous  $\text{Na}_2\text{SO}_4$ , evaporated to dryness under reduced pressure, and saponified with boiling saturated KOH-MeOH for 1 minute after the original color has returned. The pigments are then forced into 25 cc. of ether by dropwise neutralization with concentrated HCl. Three extractions with ether insure completeness. The combined extracts in both series are then extracted four times with 5 per cent HCl, and four times with 12 per cent. The chlorin *e* and rhodin *g* so prepared are made to volume and the transmissions are measured at 6600 Å. in the respective acid concentrations. This makes it possible to determine the rates at which chlorophylls *a* and *b* are hydrolyzed in the natural mixture.

The course of the reaction was always checked by testing the solutions in ether with respect to extraction of chlorophyllides with 22 per cent HCl, with 0.01 N NaOH in the case of the free

chlorophyllide, and both fractions, unaltered and hydrolyzed, were checked for allomerization by the phase test. Only in the case of the weak alcohol system in spinach, and at certain times with sunflowers, did the samples occasionally fail to respond positively.

The variability of the enzyme systems in the three solvents is shown in Table I.

TABLE I  
*Per Cent Hydrolysis of Chlorophyll*

Leaf source	Time	Ethanol, in 24 hrs.*	Acetone, in 24 hrs.*	Hot water, in 20 min.	Remarks
<i>Spinacia</i>	Mar.	Trace	80	100	From market
	May	None	10	5	" "
	Aug.	"	20	75	" "
	Nov.	68 in 27 hrs.	58 in 27 hrs.	31.5	" "
<i>Helianthus</i> †	July	75	25	50	Manteca
	Oct.	50	90	30	"
	Aug.	90		50	Palo Alto
	Sept.	75	100 in 1 hr.	100	Berkeley
<i>Heracleum</i>	Mar.	90	100	10	Berkeley Hills
	May	58 in 2 hrs.	71 in 2 hrs.	20	Berkeley Hills
<i>Scrophularia</i>	"	100	25	None	Mature
	"	78 in 2 hrs.	76 in 2 hrs.	"	Mature, frozen
<i>Avena</i>	Dec.	60 " 3 "	54 " 3 "	"	Young
	Spring	None	None	"	At all stages
	Summer	"	"	"	of growth

\* Except where otherwise indicated.

† From one lot of seed.

Varietal differences possibly account for some of the spinach variations, but this factor is excluded from the other cases. The results suggest that two and possibly three different systems are involved, but this is not easily proved. The purified enzyme systems are much weaker, and the rate constant falls rather rapidly with time, so that the systems are not readily differentiated. Chlorophyll forms part of a pigment-protein complex, chloroplastin (4), in the leaf. While the primary effect of all three solvents must be to denature the chloroplastin, the organic solvents

also cause a disintegration of the plastids, whereas the hot water does not. We have elsewhere (5) disagreed with the view that hot water ruptures the chloroplasts. The system functioning in hot water in all probability therefore attacks a denatured chloroplastin, whereas the acetone and alcohol systems attack an extracted chlorophyll. This may be considered proved by the fact that the substrate can be extracted in ethanol or acetone and recombined with the enzyme to undergo hydrolysis. In the former instance, the evidence is less certain. Anson (6) considers the chlorophyll-protein complex isolated with digitalin to be denatured. Its absorption agrees with that of the leaf treated with hot water, maximum 6750 Å.; cf. 6820 Å. for the living leaf. The evidence that chlorophyll goes into solution in the lipid

TABLE II  
*Effect of Freezing and Drying on Enzyme Activity*

Source	System	Per cent hydrolysis	Treatment
Sunflower	Acetone (2 hrs.)	79	Fresh
		81	Frozen
		27	Dried
<i>Heracleum</i>	Water, 75° (20 min.)	5	Fresh
		20	Frozen
Spinach	" 75°	95	Fresh
	" 50°	10	"
	" 50°	90	Frozen

fraction of the plastid is becoming therefore increasingly a matter of supposition.

Inactivation temperatures are different, but here again the problem of accessibility of substrate to enzyme is involved, so that we have no conclusive answer, particularly in contrasts between behavior in acetone and in alcohol.

The effect of pretreatment of the leaf on enzyme activity is shown in Table II.

Immersion of the leaf in boiling water for 1 minute is sufficient to destroy the water system. The acetone system shows comparable sensitivity, but the alcohol system is more resistant in the case of figwort. This is probably because of the high activity of the enzyme in this plant, particularly in the spring.

Ethyl chlorophyllide crystals may be obtained from leaves after a pretreatment of 5 minutes at 100°. As this leaf contains no water system, there is no hydrolysis prior to placing the leaf in alcohol. Addition of electrolytes is necessary to demonstrate activity after longer periods, and 10 minutes usually suffice for complete inactivation regardless of subsequent additions.

Formaldehyde, KCN, and NaF had little effect on the activity of the *Scrophularia* enzyme in alcohol, but  $\text{Hg}(\text{NO}_3)_2$  caused complete inactivation in a concentration roughly 0.1 per cent.

*Rates of Reaction*—Willstätter and Stoll (2) indicate, in spite of a certain falling in the values of the rate constant with time, that the reaction is of the first order. However, even with their own data ((2) p. 186) there is little to choose between first and second order rate constants. They observed (7) that hydrolysis obeyed Schütz's rule. With a constant quantity of enzyme, in any given experiment, we have found a remarkably constant value for  $k$  in the expression,  $x = k \sqrt{t}$ , up to values of  $x$  (the amount hydrolyzed in time  $t$ ) of well over 60 per cent. The conclusion is drawn (8) that in such cases the reaction velocity is not only proportional to the substrate concentration existing at the time, but is also inversely proportional to the quantity already transformed. The reaction does not take place in homogeneous solution, and there is marked reduction in activity on purification, so that there is at present too much uncertainty in the application of the Michaelis-Menten equation to our data.

In Table III, the enzyme has been permitted to work on its own native substrate *in situ* in the leaf or leaf powder. The results with figwort show that chlorophyll *a* is hydrolyzed more rapidly than chlorophyll *b*, by 10 to 20 per cent in ethanol, by 20 to 50 per cent in the case of acetone. The constant  $k_1$  is based on the usual first order reaction equation ( $t$  in hours, logarithm to the base  $e$ ); inasmuch as the natural substrates were used and only relative concentrations determined for most of the data,  $k_2$  which has dimensions of  $1/c$  was calculated upon the arbitrary assignment of 100 for  $c$ , the concentration. The  $k$  from Schütz's rule is similarly defined, in terms of  $x$  on a percentage basis, with  $t$  in hours. The chlorophyll mixture was determined in the case of *Heracleum* and spinach, while with figwort, the two components were measured separately, as already described.

Attempts at purification have so far always reduced enzyme activity. A pigment-free enzyme meal was obtained by washing frozen ground figwort with acetone. To equal quantities of the meal were added pure chlorophylls *a* and *b*, in acetone (about 200 mg. per liter). This was then made to 70 per cent acetone with an aqueous leaf extract to serve as a source of electrolytes. After 12 hours, 70 per cent of the chlorophyll *a* was hydrolyzed,

TABLE III  
*Rate Constants for Chlorophyllase Activity*

Source	Time	Hydrolysis (chlorophyll <i>a</i> + <i>b</i> )		$k_1 \times 10^3$	$k_2 \times 10^3$	$k$ (Schütz)	
	<i>min.</i>	<i>per cent</i>					
<i>Heracleum</i> , dried, in ethanol	15	12.5		534	5.71	25	
	30	17.5		383	4.24	24.7	
	60	25		288	3.33	25	
	90	31		247	3.00	25.3	
<i>Spinacia</i> , fresh, in water, 75°	1	50		41,600	600	387	
	3	75		27,700	600	335	
	5	80		19,300	480	314	
	10	85		11,400	340	208	
		<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
<i>Scrophularia</i> , frozen, in ethanol	15	34	30	1680	1417	68	60
	30	52	44.5	1473	1178	73.5	62.8
	60	64	57	1023	846	64	57
	120	78	66.5	754	547	55	47
<i>Scrophularia</i> , frozen, in acetone	30	47	31	1270	743	66	43.8
	60	64	44	1023	580	64	44
	120	76	60	715	459	53.6	42.3
	240	85.5	73	482	327	42.8	36.5

38.6 per cent of the chlorophyll *b*, a ratio of 1.81:1, identical with Mayer's values (3).

#### SUMMARY

Chlorophyll may be enzymatically hydrolyzed in three solvents, for which optimal conditions in a period varying from some minutes to 24 hours are as follows: ethanol, 80 per cent, 25°; acetone, 40 to 70 per cent, 25°; water, 75°. Virtually no activity is found with wild oats; spinach shows at times high activity in



water, but little in acetone or alcohol; figwort shows extremely high activity in alcohol, but none in water; numerous other plants investigated show intermediate activities. The enzyme is difficult to extract, and best results with enzyme meal indicate at least a 50 per cent reduction in activity.

The evidence indicates that the enzyme acts on chlorophyll in the case of 80 per cent alcohol, whereas in the water system, the chlorophyll is still within the plastid, and may be merely a denatured chloroplastin. The acetone shows some inconsistencies, and it is difficult to determine whether the upper limit for acetone (70 per cent or more) is in effect the system operating in alcohol, and the lower limit (30 to 40 per cent) caused by the water system. The acetone system in sunflowers particularly requires further investigation in this respect. At certain seasons, spinach may show no hydrolysis in ethanol, and yet be completely hydrolyzed in hot water in 20 minutes; the reverse is true for figwort. Spinach contains no natural inhibitor, as crystal formation could readily be induced with figwort enzyme meal.

Much of the variability in all instances must be due to the inaccessibility of the chlorophyllase to the substrate, and the unusual conditions under which the enzyme operates must be, in large measure, conditions which render the substrate accessible.

The preparation of pure chlorophyll with intact phytol requires us to take cognizance of a system capable of hydrolyzing 80 per cent of the chlorophyll in a sunflower leaf in half an hour, in acetone.

The reaction constants for first and second order equations show marked falling off with time. The constant based on Schütz's rule, however, shows much less variation.

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## THE EFFECT OF DIET ON THE PANTOTHENIC ACID CONTENT OF CHICK TISSUES

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In general the vitamin content of tissues (combined or uncombined) decreases during vitamin deficiency. Thus Ochoa and Peters (1) found that cocarboxylase decreased in tissues of pigeons fed diets deficient in vitamin B<sub>1</sub>. Axelrod and Elvehjem (2, 3) reported a diminution in the cozymase content of liver and muscle of dogs and pigs fed nicotinic acid-deficient rations. No effect, however, was noted in the coenzyme content of brain, kidney cortex, or blood. Vilter *et al.* (4) and Kohn (5) have shown a decrease in the factor V (coenzyme I + coenzyme II) content of the blood of pellagrins which could be remedied by administration of nicotinic acid. A similar decrease in the flavin and flavin-adenine dinucleotide content of tissues from rats fed diets deficient in riboflavin has been reported by Axelrod, Sober, and Elvehjem (6) and by Ochoa and Rossiter (7).

The recent demonstration of the essential nature of pantothenic acid for the chick (8, 9) makes it of interest to determine to what extent such diminutions in the pantothenic acid content of chick tissues occur on diets deficient in this substance. The following investigation was made for this purpose.

### EXPERIMENTAL

Day-old white Leghorn chicks were fed a commercial starting diet for 5 days. They were then placed in two separate brooders. Group 1 (twenty-two chicks, average weight 59 gm.) was fed the heated (pantothenic acid-deficient) diet of Jukes (10); Group 2 (fifteen chicks, average weight 57 gm.) was fed the same ration

supplemented with a pantothenic acid concentrate from liver. This preparation had a "potency" (11) of 600, and was fed at a level of 30 mg. per 100 gm. of diet. Jukes (12) found this level necessary for a maximum rate of growth for chicks on this diet (assay of a commercial starting mash showed a pantothenic acid content about 1.3 times this high). Individual weight records were kept of all chicks. Differences in the average weight of members of the two groups became apparent from the first weighing (after 3 days) on; after 19 days chicks on the deficient diet averaged 85 gm., those on the sufficient diet averaged 165 gm. Symptoms (scabbed beaks and eyes, cracked feet) were moderate to severe in the deficient group. Several chicks from each group were killed and portions of the brain and spinal cord analyzed for pantothenic acid. After a further 7 days, the remaining chicks on the deficient diet averaged 99 gm. and showed severe symptoms; those on the sufficient diet averaged 215 gm. and appeared normal. Another set of checks from each group was sacrificed at this time, and analyses made for the pantothenic acid content of leg muscle, liver, kidney, and blood.

*Analytical Methods*—Tissue samples were taken immediately after death; the tissues were weighed intact and then homogenized by the method described by Potter and Elvehjem (13). They were then suspended in 6 to 10 volumes of water and autolyzed under benzene for 24 hours at 37°. Autolysis has been shown (14) to liberate additional pantothenic acid from a "bound" water-insoluble form which occurs in tissues. After autolysis samples were steamed to remove the benzene; the coagulum was filtered out, washed, and the combined filtrates diluted to a suitable volume. Blood was collected in sufficient citrate to prevent coagulation; red cells were laked by diluting 10-fold with water and overlaying with ether for 6 hours; samples were then heated and filtered.

The pantothenic acid content of all extracts was determined by the yeast test method of Williams *et al.* (11, 15, 16), and by a modification of the bacterial test of Snell *et al.* (17, 18) which will form a separate communication from this laboratory. In both methods the growth response elicited by aliquots of the extract is compared with that obtained from a purified pantothenic acid

preparation of known potency. The comparison is made at different dosage levels; agreement of assay values at these different levels is an indication of the specificity of the test (*cf.* (19, 20)).

TABLE I

*Comparison of the Pantothenic Acid Content of Chick Tissues As Determined by Yeast and Bacterial Methods*

Tissue	Extract assayed equivalence in tissue	Pantothenic acid found		Pantothenic acid per mg. tissue	
		Yeast	<i>Lactobacillus casei</i> $\epsilon$	Yeast	<i>Lactobacillus casei</i> $\epsilon$
	mg.	mg. units	mg. units	mg. units	mg. units
Brain (sufficient diet)	0.5	0.27	0.26	0.54	0.52
	1.0	0.60		0.60	
	1.5		0.86		0.57
Average.....				0.57	0.55
Brain (deficient diet)	0.5	0.12	0.13	0.24	0.26
	1.0	0.20		0.20	
	1.5		0.30		0.20
	2.0	0.38		0.19	
	4.0		0.84		0.21
Average.....				0.21	0.22
Leg muscle (sufficient diet)	0.5	0.20	0.17	0.40	0.34
	1.0	0.30	0.33	0.30	0.33
	2.0	0.84	0.64	0.42	0.32
Average.....				0.37	0.33
Leg muscle (deficient diet)	1.0	0.07		0.07	
	2.0	0.10	0.26	0.05	0.13
	4.0	0.21	0.51	0.05	0.13
	8.0		0.81		0.10
Average.....				0.06	0.12

When this and other criteria are applied, the bacterial test appears more reliable for use with crude tissue extracts. Both tests give identical results when applied to purified preparations of pantothenic acid.

*Results*

Detailed results of assays by both methods on two tissues are given in Table I. The results shown in Tables II and III were similarly obtained; average values only are given to conserve

TABLE II  
Set 1. Pantothenic Acid Content of Chick Tissues  
(Mg. Units per Mg. of Tissue)

Chick No.*	Weight when killed	Brain		Spinal cord	
		Yeast	<i>Lactobacillus casei</i> ε	Yeast	<i>Lactobacillus casei</i> ε
	gm.				
27 (+)	174	0.57	0.70	0.60	0.78
28 (+)	150	0.61	0.49	0.54	0.58
39 (+)	147	0.57	0.55		
26 (-)	102	0.21	0.22	0.37	0.19
29 (-)	86	0.30	0.25		
37 (-)	93	0.24	0.28		
39 (-)	55	0.23	0.30	0.30	0.22
46 (-)	95	0.20	0.28		

\* (+) indicates a chick of the group on the sufficient diet; (-) indicates a chick from the deficient group.

TABLE III  
Set 2. Pantothenic Acid Content of Chick Tissues  
(Mg. Units per Mg. of Tissue)

Chick No.*	Weight when killed	Liver		Kidney		Leg muscle		Blood	
		Yeast	<i>Lactobacillus casei</i> ε	Yeast	<i>Lactobacillus casei</i> ε	Yeast	<i>Lactobacillus casei</i> ε	Yeast	<i>Lactobacillus casei</i> ε
	gm.								
32 (+)	209	0.72	0.93	0.75	0.80	0.27	0.21		0.0023
35 (+)	223	0.85	1.08	0.57	0.82	0.27	0.25	0.002	0.0046
36 (+)	212	0.74	1.19	0.56	0.83	0.35	0.34	0.002	0.0053
49 (+)	207	0.93	0.89	0.76	0.88	0.37	0.33		
32 (-)	73	0.24	0.49	0.38	0.65	0.09	0.11	0.000	0.0011
41 (-)	101	0.31	0.62	0.33	0.54	0.07	0.11		
43 (-)	95	0.37	0.66	0.40	0.50	0.06	0.12	0.000	0.0010
49 (-)	75	0.23	0.75	0.33	0.59	0.05	0.12	0.000	0.0012

\* (+) indicates a chick of the group on the sufficient diet; (-) indicates a chick of the deficient group.

space. In Table IV the pantothenic acid contents of tissues from chicks on deficient and sufficient diets have been pooled, averaged, and compared with one another on the basis of "mg. units" (11) per mg. of tissue, and mg. units per entire tissue. The former procedure minimizes the actual difference between groups; this is emphasized by the latter method.

TABLE IV

*Pantothenic Acid Content of Chick Tissues; Collected Results*

All figures for the pantothenic acid content of tissues were calculated from data secured by the bacterial (*Lactobacillus casei* ε) method.

	Diet*	Liver	Kidney	Leg muscle	Blood	Brain	Spinal cord
No. of chicks assayed	+	4	4	4	3	3	2
	-	4	4	4	3	5	2
Average weight of chicks assayed, gm.	+	213	213	213	215	157	162
	-	86	86	86	81	86	78
Average weight of tissues assayed, gm.	+	6.38	2.80	4.66		1.68	0.38
	-	2.77	1.04	1.16		1.41	0.27
Average pantothenic acid content per mg. tissue, mg. units	+	1.02	0.83	0.28	0.0041	0.58	0.68
	-	0.63	0.57	0.115	0.0011	0.27	0.21
Pantothenic acid content per average complete tissue, mg. units	+	6510	2325	1300		975	260
	-	1750	595	133		380	57

\* The plus sign indicates that chicks on the sufficient diet were used; the minus sign, those on the deficient diet.

## DISCUSSION

Both assay methods agree in showing that in every tissue tested from chicks raised on the deficient diet the pantothenic acid content was markedly lower than in the corresponding tissue from chicks fed the supplemented diet.

Because of its greater reliability with crude extracts, results secured by the bacterial assay have been used in calculating the data in Table IV. Examination of these data shows that deficient tissues contained from 27 (blood) to 68 per cent (kidney) as much pantothenic acid as the normal tissues per gm. On the basis of total weight of tissues produced, deficient tissues con-

tained from 10 (leg muscle) to 38 per cent (brain) as much pantothenic acid as the control tissues. The low content (31 per cent of normal) in spinal cord from deficient chicks is interesting in view of the findings of Phillips and Engel (21) that specific lesions appear in this tissue in the absence of pantothenic acid which can be prevented by its addition to the diet.

We wish to express our thanks to Dr. T. H. Jukes for kindly providing us with a supply of his heated ration, and to the Rockefeller Foundation for grants in support of the investigation.

#### SUMMARY

Assays of tissues from chicks grown on diets deficient and sufficient in pantothenic acid by both yeast and bacterial methods show that in the deficient state the tissues contain only 30 to 70 per cent as much pantothenic acid per gm. as corresponding tissues from "sufficient" chicks. Since deficient chicks are much smaller than normal, the absolute pantothenic acid content of entire tissues of the deficient chicks is only 10 to 40 per cent that of normal tissues.

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## THE ISOLATION OF $\alpha$ -DIHYDROTHEELIN FROM HUMAN PLACENTA

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Subsequent to Fellner's (1912) pioneer research showing that injection of lipid extracts of human placenta produces growth in the rabbit uterus, several investigators (Frank and Rosenbloom, 1915; Giesy, 1920; Frank, Gustavson, *et al.*, 1926) studied the purification of the hormone of the placenta. Progress in the purification was facilitated by the introduction of the vaginal smear method of assay by Allen and Doisy (1923), and in a short time Laqueur *et al.* (1927) obtained excellent estrogenic preparations from placenta.

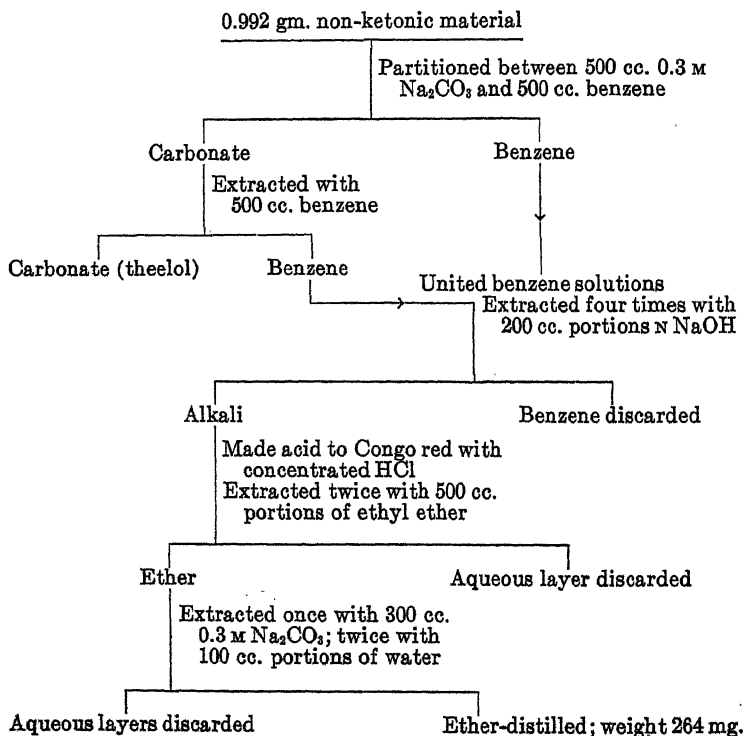
Following the discovery of the high concentration of estrogens in the urine of pregnant women (Aschheim and Zondek, 1927), the work on placenta languished, and it was not until 1931 that Browne, working with Collip, isolated the first crystalline estrogenic compound from placenta. This compound was later found to be identical with theelol (Butenandt and Browne, 1933) which had previously been isolated from urine (Marrian, 1930; Doisy *et al.*, 1930). Collip and coworkers (1930) reported the presence of a water-soluble ether-insoluble substance (emmenin) in placenta, but this constituent has never been actually identified. However, there is reason for believing that it may be a glucuronide of theelol (Cohen and Marrian, 1936).

In 1938, Westerfeld, MacCorquodale, Thayer, and Doisy separated the non-ketonic from the ketonic estrogens of human placenta and showed that the latter is mainly, if not entirely, theelin. The present work consists of a study of the non-ketonic fraction obtained in the previous work.

## EXPERIMENTAL

The extraction of 422 kilos of human placenta, purification of the extract, and the separation of the ketonic fraction have been described by Westerfeld *et al.* (1938). The non-ketonic fraction weighing 1.983 gm. was a dark red oil containing some crystals. Since this material was so valuable, because so much labor had been involved in its preparation, it was divided into two equal portions for purification. However, the processes worked so well that no difficulty was encountered in isolating and identifying dihydrotheelin in the 0.992 gm. used.

The flow sheet gives the procedure followed to separate the dihydrotheelin from most of the contaminating substances. According to Dr. Alan Mather (unpublished work) the separation of theelol from dihydrotheelin by distribution between benzene and 0.3 M sodium carbonate is almost quantitative, the former



passing almost entirely into the aqueous phase. Assuming that only these two estrogens were present, the assays of the two fractions indicated the presence of 30 mg. of theelol and 8 mg. of dihydrotheelin.

*Isolation of  $\alpha$ -Dihydrotheelin*—The 264 mg. of material containing the dihydrotheelin were dissolved in a mixture of 30 cc. of *n*-butyl alcohol and 150 cc. of petroleum ether (b.p. 30–60°). This solution was extracted ten times with 40 cc. portions of 0.25 N NaOH. Hydrochloric acid was used to acidify the solution, which was then extracted twice with ether. The ether was washed twice with 0.3 M  $\text{Na}_2\text{CO}_3$  and twice with water. After the combined carbonate and water layers had been united, they were extracted once with ether, which was then washed with carbonate and water. The ether solutions were combined and distilled to dryness, yielding a semicrystalline residue.

The semicrystalline material was dissolved in 100 cc. of 0.25 N NaOH, the alkalinity adjusted with HCl until the solution was just red to phenolphthalein, and the alkaline solution approximately saturated with solid NaCl. A series of ten extractions with 50 cc. portions of ether was employed to regain the estrogen; then the ether was thoroughly washed with water and distilled.

The residue from the ether distillation was partitioned between toluene and 0.3 M  $\text{Na}_2\text{CO}_3$ ; the toluene layer was then treated in the same fashion as the benzene, as given in the flow chart. The yield was 23 mg. of a glassy, yellowish brown material which, according to the assay, contained 5 mg. of dihydrotheelin.

The crude estrogen was naphthoylated by the method previously described by MacCorquodale, Thayer, and Doisy (1936), and the crude naphthoate crystallized from 95 per cent alcohol. Altogether, two treatments with norit, three recrystallizations from alcohol, and two recrystallizations from acetone were employed to purify the di- $\alpha$ -naphthoate. In spite of this lengthy purification, 6.80 mg. ( $\approx$  3.08 mg. of dihydrotheelin) of the pure  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate were obtained. The melting point was 194–195° (corrected), a mixed melting point with authentic  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate (m.p. 197–197.5°, corrected) being 195–196° (corrected).

*Microcombustion Analysis*— $\text{C}_{40}\text{H}_{36}\text{O}_4$

Calculated, C 82.72, H 6.25; found, C 82.70, H 6.35

A portion of the  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate was hydrolyzed with KOH in aqueous alcohol. The phenol was recovered and crystallized from aqueous alcohol after having been treated with a small amount of norit, m.p. 172–172.5° (corrected); a mixed melting point with authentic  $\alpha$ -dihydrotheelin (m.p. 174–175.5°, corrected) was 174–175° (corrected).

The  $\alpha$ -dihydrotheelin isolated from placenta was assayed against the authentic specimen of  $\alpha$ -dihydrotheelin with the precautions customarily observed in our work. Of twenty-four rats injected with 0.10  $\gamma$  of the placental product sixteen gave a positive response; seventeen of the twenty-four injected with authentic dihydrotheelin gave a positive response.

#### SUMMARY

1. Bioassay results on an extract of 422 kilos of human placenta show that the approximate quantities of estrogenic materials are theelol 0.14, theelin 0.035, and dihydrotheelin 0.038 mg. per kilo of tissue.

2. A phenol which proved to be  $\alpha$ -dihydrotheelin was isolated from the non-ketonic fraction of human placenta.

We wish to express our thanks to Dr. W. W. Westerfeld for his donation of the non-ketonic fraction of the estrogens of 422 kilos of human placenta. We also desire to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

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## THE FAILURE OF INTRAVENOUSLY INJECTED FAT TO PRODUCE CHOLIC ACID IN THE DOG\*

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Whipple and his coworkers (1) reported several years ago that the taurocholic acid excretion of the dog was probably related to protein metabolism. These workers fasted bile fistula dogs and found a low taurocholic acid concentration in the bile, which decreased still more when carbohydrate was fed (2). This was attributed to a protein-sparing action of the carbohydrate, which made less protein available for bile acid synthesis by decreasing endogenous protein metabolism. Feeding high protein diets (3) caused a rise in bile salt output, as did alcohol-extracted meat. A high cholesterol diet, fed as egg yolk, failed to produce extra bile salt excretion.

Jacobi, Zuckerman, and Kogut (4) have recently reviewed the literature with respect to bile excretion on carbohydrate, protein, or fat diets. Most of the experiments described measured only bile volumes. The experiments of Whipple (1-3) were the only ones in which bile salt excretion was determined.

It is generally accepted that bile salts play a part in the absorption of fat (5). If an animal has a bile fistula, it is obvious that the fats of its diet cannot be normally absorbed. Whipple's experiments measuring the power of an animal to produce bile salts from high fat diets cannot be interpreted quantitatively, for no measure of the fat actually reaching the blood stream is available. To overcome this difficulty, injection of fat directly

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into the blood stream was undertaken. The experiments of Holt, Tidwell, and Scott (6) were used as a basis for our work.

#### EXPERIMENTAL

A few days after a female dog had undergone a bile fistula operation it was put on a starch diet. The starch was fed in suspension in water in three daily doses which were calculated to give it 50 calories per kilo per day. This amount was somewhat below a normal value but served to maintain it in good condition for the duration of the experiment. The urine and bile were collected at 24 hour intervals, just before one of the feedings. After 4 days on the carbohydrate diet, it was put on a protein diet for 4 days. The protein consisted of casein in gelatin capsules in quantities sufficient to furnish 50 calories per kilo per day. Each capsule weighed 1 gm. and held 8 gm. of casein.

When our preliminary experiments had proved successful, we carried out further experiments on pure foodstuffs on each of three bile fistula dogs. Each animal was put on a diet of starch for 3 days, then was kept on fat for 3 days, and finally was fed protein for 3 days. The urines were analyzed for total nitrogen (Kjeldahl) and the bile for cholic acid according to the method of Mellanby and Suffolk (7). The starch and protein were fed as in our preliminary experiment. The fat was prepared as follows: Equal quantities of commercial egg lecithin and olive oil were stirred in water to make a 7 per cent emulsion of fat. The mixture was then homogenized at 4000 pounds pressure. It was sterilized at 15 pounds pressure and made isotonic by adding 3 per cent of its volume of a sterile 30 per cent solution of sodium chloride just before injection. The fat particles averaged slightly less than 1 micron in diameter. This fat suspension was injected intravenously into the animals in amounts that afforded 50 calories per kilo per day. The daily administrations were divided into three injections each. The dogs lay very quietly without anesthetic while the fat suspensions were run into the small saphenous vein from a pressure bottle. Each injection required about 15 minutes.

#### *Results*

Table I presents the nitrogen output and cholic acid production of Dog 54 on the preliminary experiment, and of Dogs 54, 57, and

TABLE I

*Cholic Acid and Nitrogen Eliminated by Bile Fistula Dogs Maintained on Diets of Pure Carbohydrate or Protein, or on Intravenously Injected Fat*

Dog No.	Day of experiment	Nitrogen	Cholic acid	Bile volume	Food during previous 24 hrs.		Weight
		gm.	mg.	cc.	gm.		kg.
54	Start						12.3
	1	6.34	1089	159	150	Potato starch	
	2	3.53	485	100	150	" "	
	3	3.26	383	75	150	" "	
	4	2.99	340	66	150	" "	11.6
	5	14.22	504	141	150	Protein	
	6	24.08	713	181	150	"	
	7	24.16	733	211	150	"	
	8	Urine lost	1009	222	150	"	11.0
54	Start						12.7
	1	7.61	1184	225	150	Potato starch	
	2	3.98	685	141	150	" "	
	3	2.76	372	105	150	" "	12.3
	4	3.76	211	120	70	Fat intravenously	
	5	3.30	173	112	70	" "	
	6	4.56	36	25	70	" "	11.9
	7	10.03	395	150	150	Protein	
	8	21.02	736	181	150	"	
57	Start						11.6
	1	11.79	2919	104	250	Potato starch	20.0
	2	6.86	1932	48	250	" "	
	3	8.42	1613	55	250	" "	19.0
	4	11.80	883	53	116	Fat intravenously	
	5	6.08	867	52	116	" "	
	6	6.18	350	28	116	" "	18.4
	7	9.97	2486	139	250	Protein	
	8	37.34	4729	187	250	"	
58	Start						17.9
	1	34.91	6092	180	250	"	20.0
	1	4.12	1627	106	175	Potato starch	
	2	3.78	1920	90	175	" "	
	3	3.32	1511	79	175	" "	14.3
	4	3.30	Chewed balloon		79	Fat intravenously	
	5	3.07	438	78	79	" "	
	6	4.05	503	85	79	" "	14.2
	7	20.57	1841	154	175	Protein	
	8	19.34	3434	192	175	"	
	9	27.62	4521	187	175	"	14.1

58 for the fat injection experiments. An examination of the figures for nitrogen elimination will show that the output dropped steadily when protein was not present in the diet. As might have been expected, the nitrogen excretion rose when rather large quantities of protein were fed. A lag period was noted both when protein was eliminated from the diet, and when a period of protein feeding was started.

The values obtained after injection of fat indicate that no cholic acid production was stimulated by fat. To be certain that the dogs had utilized the injected fat, ether extracts were made of both the bile and urine of Dogs 57 and 58 following fat administration and carbohydrate feeding. The residues left after evaporation of the ether were weighed. No more solids per cc. of bile were obtained after the fat injections than after carbohydrate feeding. As the volumes of bile after fat administration were smaller than those obtained after carbohydrate feeding, the total daily output of ether-soluble materials was less following injections of fat than on diets of carbohydrate. This indicates to us that no appreciable quantities of fat escaped utilization by way of bile or urine.

Each time carbohydrate was fed, the cholic acid excretion diminished, which is in accord with Whipple's results. When fat was administered for 3 days, immediately following the period of carbohydrate feeding, the cholic acid excretion diminished still more. This diminution was not due to a loss of ability of the dog to form cholic acid, for on the following days on which protein was fed the cholic acid output rose to values which were greater than those observed on the first days of the experiments. It will also be noted that while the volume of bile was small on the carbohydrate and fat régimes and large on the protein diet, the cholic acid elimination was not proportional to the volume of bile.

#### DISCUSSION

In the experiments described there was no elimination of fat either in bile or in urine. The fat was put directly into the blood stream. Gordon and Levine (8) have presented data which suggest that fat introduced by vein is promptly burned. The evidence seems to warrant the belief that there is high probability that fat introduced intravenously is utilized. This apparent utilization of fat which definitely reached the blood stream is the

outstanding difference between our experiments and those of Whipple. Our results are quite in accord with his which were obtained by oral administration of fat. We must simply further emphasize his conclusion; namely, that protein rather than fat or carbohydrate is the material from which the body forms cholic acid.

#### SUMMARY

Bile fistula dogs were maintained for consecutive 3 day periods on a carbohydrate diet, on intravenously injected fat emulsions, and on a protein diet. Each type of food furnished 50 calories per kilo per day. The cholic acid excretion on the carbohydrate and fat régimes decreased steadily, whereas on the protein diet it rose to high values.

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## THE PRESERVATION OF ASCORBIC ACID IN DRAWN SAMPLES OF BLOOD\*

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In clinical or experimental studies of blood ascorbic acid, it is obviously important to maintain the ascorbic acid at its original level until analysis has been made. Pijoan, Townsend, and Wilson (1) reported a loss of about one-half of the vitamin C of blood plasma when the latter stood for 90 minutes at room temperature. Pijoan and Klemperer (2) recommended the addition of 1 mg. of KCN per cc. of blood as a preservative of ascorbic acid. Friedman, Rubin, and Kees (3) observed no loss in plasma ascorbic acid in 160 minutes at ice box temperature and reported the use of KCN to be unnecessary. Farmer and Abt (4) reported that KCN does not prevent loss of ascorbic acid in blood and that its use is objectionable, because it gives enhanced values owing to the decolorizing action of KCN upon 2,6-dichlorophenol indophenol. Cushman and Butler (5) observed a variation in the decolorization of indophenol by KCN, depending upon the lot of KCN used, the pH of the reaction, and the concentration of KCN. These authors found that the use of KCN in the amounts recommended by Pijoan and Klemperer does not necessarily invalidate the results, but that there is no advantage in its use.

We have made studies of the stability of ascorbic acid in blood, using the method of Mindlin and Butler (6) for its determination. In agreement with Pijoan, Townsend, and Wilson (1) we found a steady decrease in the ascorbic acid of plasma removed from the red cells when allowed to stand at room temperature. We attempted to prevent this loss by the use of antioxidants which

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do not reduce indophenol, such as hydroxyquinoline, orcinol, resorcinol, salicylic acid, and maleic acid, without success. We next turned our attention to a study of the natural preservative system in blood which prevents the oxidation of ascorbic acid.

The presence in blood of substances that inhibit the catalytic oxidation of ascorbic acid has been reported by De Caro and Giani (7), Kellie and Zilva (8), and Barron, Barron, and Klemperer (9).

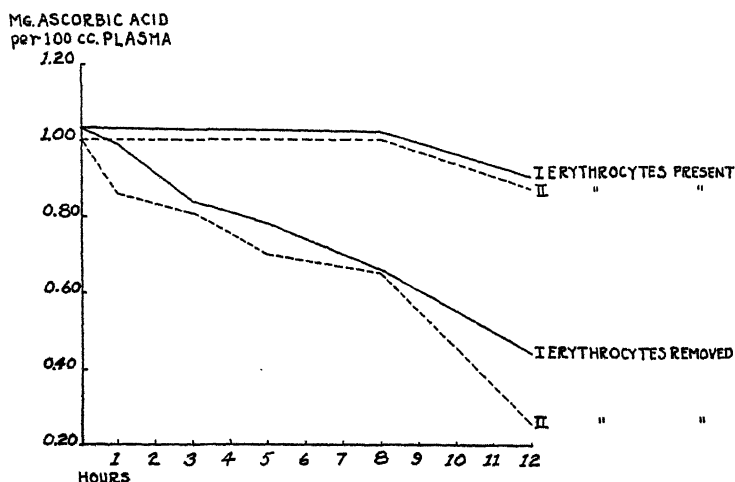


FIG. 1. Effect of erythrocytes on the stability of plasma ascorbic acid. Curves for Subjects I and II.

In 1936 Roe and Barnum (10) reported the presence of a substance in blood which reduces dehydroascorbic acid. Borsook, Davenport, Jeffreys, and Warner (11) observed an *in vitro* reduction of dehydroascorbic acid by kidney, liver, and intestine, which they suggested was due to the action of glutathione. The data of Borsook *et al.* show a reduction of dehydroascorbic acid by plasma and whole blood, but these authors attribute this to a non-ascorbic acid indophenol-reducing substance and they deny the existence of a mechanism in blood for reducing dehydroascorbic acid. Schultze, Stotz, and King (12) observed an *in vitro* reduction of dehydroascorbic acid by suspensions of liver, muscle, small intestine, blood, and erythrocytes. These authors

attribute the reduction of dehydroascorbic acid by tissues to the action of glutathione and fixed —SH compounds.

In view of these reports showing an antioxidant mechanism for ascorbic acid in the blood, it seemed that an effective preservation of the preexisting ascorbic acid of blood might be obtained by taking advantage of the natural protective substances present if optimum conditions were set up. Experiments such as are shown in Fig. 1 demonstrated this to be correct. In this experiment two large samples of blood were collected and divided into two portions. One portion was centrifuged, the plasma was removed at once, and a control vitamin C determination was made. These portions of plasma were then allowed to stand at room temperature along with the undisturbed samples of whole blood. Sterile conditions were maintained throughout the experiment. At intervals following the control determination, the vitamin C was determined in the segregated plasma and in a sample of plasma obtained by centrifuging a portion of the whole blood just previous to the analysis. A comparison was thus obtained of the stability of ascorbic acid in plasma in the presence and absence of red cells. The curves show a steady loss of ascorbic acid in the segregated plasma and an effective protection of the vitamin in the plasma allowed to remain in contact with the red cells.

In the experiment of Fig. 1 there was no change in the ascorbic acid content of plasma in contact with red cells in 8 hours. The preservation of ascorbic acid varies, however, in different bloods. This is shown by the data of Table I, in which the ascorbic acid content of some blood plasmas in contact with their red cells remained practically constant for 24 hours at room temperature. In other experiments we found that the ascorbic acid content of plasma kept in contact with the red cells at a temperature of about 3° remained unchanged for 52 hours.

The ascorbic acid values of blood serum that has been standing, expressed in mg. per 100 cc., are as follows:

	0 hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.
Sample 1.....	1.29	1.34	1.30	1.13	1.10
“ 2.....	1.05	0.99	0.99	0.89	0.74



In serum the ascorbic acid appears to keep for 1 to 2 hours, but thereafter it diminishes at a rate comparable to the losses in plasma separated from the red cells.

Other experiments made it clear to us that it is very important to maintain the biological intactness of the red cells. If a few

TABLE I  
*Data Showing Stability of Ascorbic Acid in Blood Plasmas Allowed to Stand in Contact with Their Red Cells*

As anticoagulant, either 1 drop of 20 per cent potassium oxalate per 5 cc. or 1 drop of 30 per cent sodium citrate per 5 cc. was used.

Sample No.	Container and anticoagulant	Ascorbic acid per 100 cc. plasma after standing			
		0 hr.	12 hrs.	16 hrs.	24 hrs.
		mg.	mg.	mg.	mg.
1	Non-Pyrex glass, Na citrate	0.82		0.75	0.65
2	" " " "	1.12		0.72	
3	" " K oxalate	1.12		0.77	0.67
4	" " " "	0.89		0.42	0.29
5	Pyrex glass, Na citrate	0.62		0.64	0.56
6	" " " "	1.24		1.10	1.07
7	Paraffin-lined tube, K oxalate	0.93	0.91		
8	" " " "	0.14	0.14		
9	" " " "	0.69	0.11*		
10	" " " "	0.77		0.58*	0
11	" " " "	0.62		0.59	0.56
12	" " Na citrate	0.26		0.25	0.23
13	" " " "	0.74		0.76	0.90
14	Collodion-lined " " "	1.16		1.16	1.10
15	" " " "	0.48		0.55	0.48
16	" " " "	0.73		0.72	0.66
17	" " " "	0.97	0.74		0.83
18	" " K oxalate	0.93		0.83	0.89

\* Hemolysis.

drops of distilled water are added to blood, producing hemolysis, there is an initial decrease in the ascorbic acid value, and the ascorbic acid content of plasma containing hemoglobin diminishes upon standing. Other hemolysis-producing factors such as vigorous shaking or hemolytic bacteria have the same effect.

Having noted that the adherence of red cells to the sides of a glass container upon standing is a preliminary step towards

hemolysis, we made a study of the effect of the surface of the container upon the stability of blood ascorbic acid. As shown in Table I, the poorest results were obtained with bloods kept in ordinary glass tubes. The ascorbic acid of bloods kept in paraffin- or collodion-lined tubes and Pyrex tubes was much better preserved than when the bloods were allowed to stand in ordinary glass tubes.

Our results show clearly that to maintain the stability of ascorbic acid in whole blood the red cells must be kept intact. If the red cells are functioning normally, they have a preservative effect upon ascorbic acid; but when the cell wall is broken and oxyhemoglobin is liberated, the plasma ascorbic acid is oxidized.

We interpret the greater stability of ascorbic acid in the presence of intact red cells as being a positive effect of the red cells. The red cells have a greater capacity for sustained reduction than plasma, because they contain relatively large amounts of reduced glutathione and they possess a powerful reducing mechanism which maintains glutathione and oxyhemoglobin essentially in their reduced forms. This explanation is supported by the work of Borsook *et al.* (11), who demonstrated the reduction of dehydroascorbic acid by glutathione, and by the report of Schultze, Stotz, and King (12), who found that the red cells rapidly reduce added dehydroascorbic acid and glutathione-free plasma does not.

As the breakdown of ascorbic acid may be taken as a sensitive indication of red blood cell integrity, the principles set forth above for the preservation of blood ascorbic acid may be construed to apply to the preservation of blood in blood banks. Certainly all factors that will prevent hemolysis are important. Scudder *et al.* (13) have reported that the shape of the container is a significant factor in blood preservation as measured by the rate of diffusion of potassium from the red cells, the more rapid diffusion being observed in the container having the larger interface area. It would follow from this that the chemical nature of the blood bank container may also be of considerable importance. We found, however, that the use of paraffin-lined, collodion-lined, and Pyrex glass containers did not appreciably alter the rate of diffusion of potassium from the red cells. Further work upon the influence of the container on the preservation of blood is being done.

## SUMMARY

1. If blood plasma is separated from the red cells and allowed to stand, its ascorbic acid content decreases.

2. The ascorbic acid content of blood plasmas kept in contact with their red cells has been observed to remain unaltered for 16 to 24 hours at room temperature and for 52 hours at ice box temperature.

3. To obtain correct plasma ascorbic acid values and to preserve the ascorbic acid in blood for a time, it is necessary to prevent hemolysis completely.

4. Intact red blood cells have a preservative effect upon the ascorbic acid of the plasma in which they are suspended. Maintenance of conditions which preserve the intactness of the red cells has been found the most effective procedure for stabilizing the ascorbic acid content of drawn samples of blood.

5. Pyrex, paraffin-, and collodion-lined tubes used as blood containers gave a better preservation of the ascorbic acid of plasma than ordinary glass containers.

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## THE STABILIZATION AND DETERMINATION OF PYRUVIC ACID IN THE BLOOD\*

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The diphosphoric ester of vitamin B<sub>1</sub> (cocarboxylase) is the coenzyme necessary for the normal catabolism of pyruvic acid in body tissues. This effect has been especially studied in brain tissue (1). In vitamin B<sub>1</sub> deficiency there is an accumulation of pyruvic acid in the blood (2). A method for the determination of blood pyruvate would therefore seem of clinical importance.

Sherman and Elvehjem (3) found that there was no disappearance of pyruvate added to blood and allowed to stand for 2 hours at 37°. However, the method used was not specific for pyruvic acid, since the total carbonyl compounds (bisulfite-binding substances) were determined. Estimating the pyruvic acid as dinitrophenylhydrazone, Wilkins, Weiss, and Taylor (4) noted a considerable loss of pyruvic acid under these circumstances. We found a significant decrease, even after 1 minute of standing at room temperature. Since the precipitation of the blood, with either a special micro blood pipette or a syringe, requires from 30 to 90 seconds, the necessity for using some stabilizing agent becomes obvious.

### *Stabilization of Blood Pyruvate by Monoiodoacetate*

We have found that sodium monoiodoacetate in a concentration of 0.2 per cent completely prevents the disappearance of pyruvic acid from the blood. This is illustrated by the following experiment. Blood was withdrawn from the cubital vein of a normal

\* This work was aided by a grant from Child Neurology Research (Friedsam Foundation).

healthy adult and divided as follows: (1) A sample was precipitated with 10 per cent trichloroacetic acid *3 minutes* after withdrawal from the vein. A pyruvic acid value of 0.83 mg. per cent was obtained. (2) Another sample was allowed to stand for an additional 8 minutes (11 minutes after withdrawal from the vein) prior to precipitation and a value of 0.30 mg. per cent was obtained. (3) To other samples of the same blood, varying amounts of sodium moniodoacetate were added *3 minutes* after withdrawal from the vein. These latter samples were then precipitated after an additional 8 minutes (11 minutes after withdrawal from the vein) and pyruvic acid values of 0.82 to 0.83 mg. per cent were obtained. The moniodoacetate concentrations varied between 0.2 and 2 per cent.

Other experiments showed that if the concentration of added moniodoacetate was 0.1 per cent or less complete stabilization of pyruvic acid did not occur.

Furthermore, pyruvic acid determinations on blood samples introduced directly into a flask containing sodium iodoacetate or blood added to a flask of trichloroacetic acid with immediate precipitation yield similar results.

If the blood to which sodium iodoacetate is added is allowed to stand at room temperature for 30 minutes, an increase in pyruvic acid of 3 to 20 per cent is observed. After 3 minutes, on the other hand, no significant change occurs (Table I). Since the entire procedure (drawing of the blood and its subsequent precipitation) requires at the most 2 minutes, the possibility of any increase in such a time interval is negligible.

In addition, if blood is precipitated at intervals ranging from 40 seconds to 10 minutes after withdrawal from the vein, significant losses are observed when the stabilizing medium is not used (Table II).

### *Method*

For the determination of pyruvic acid (already stabilized by sodium iodoacetate) in the filtrate the method of Lu (5) was modified in the following ways. (1) Larger amounts of blood are used. The smaller amounts suggested by Lu fall in a colorimetric range which greatly magnifies any possible errors in reading. (2) The dinitrophenylhydrazone of the pyruvic acid, together

with the free hydrazine, is extracted first with 4 ml. and then twice with 2 ml. of ethyl acetate. This is necessary because a larger volume of filtrate is used. (3) 2N sodium hydroxide is added

TABLE I

*Effect of Standing at Room Temperature with Sodium Monoiodoacetate on Pyruvic Acid Content of Blood*

The results are expressed in mg. per cent.

Blood with 0.5 per cent $\text{CH}_2\text{ICOONa}$ ; pptd. after	Sample No.								
	1	2	3	4	5	6	7	8	9
90 sec.	1.10	0.93	0.94	1.02					
2 min.					1.11	0.96	0.90	0.99	0.83
3 "	1.10	0.96					0.90		
5 "					1.10		0.92	0.97	
7 "	1.13			1.08					
10 "						0.97			
12 "	1.16								
30 "	1.23	1.10	1.01	1.18	1.14	1.03	0.97	1.05	0.88

TABLE II

*Decrease of Blood Pyruvate on Standing; Blood Drawn without Sodium Monoiodoacetate*

	Pyruvic acid, mg. per cent		
	Sample 1	Sample 2	Sample 3
5 ml. blood caught directly in flask containing 25 mg. $\text{CH}_2\text{ICOONa}$ ; pptd. after 90 sec.....	0.94	0.97	1.03
Blood taken in syringe (no $\text{CH}_2\text{ICOONa}$ ); pptd. after			
40 sec.....			0.90
60 ".....		0.77	
4 min.....	0.51		
6 ".....	0.38		
10 ".....	0.28		

to the sodium carbonate extract instead of N sodium hydroxide. The color developed under this condition is more stable, and no interference of other keto acids is observed in blood of normal subjects.

*Procedure*—A solution of 50 per cent iodoacetic acid in water is adjusted with sodium hydroxide to pH 7.8; a measured volume corresponding to 25 mg. of iodoacetic acid (in general 0.1 ml. of a 25 per cent solution of iodoacetate) is transferred to a bottle containing 20 mg. of dried potassium oxalate. About 5 ml. of blood obtained by venipuncture are caught directly in this bottle. 3 ml. of this sample are then transferred drop by drop into an Erlenmeyer flask containing 12 ml. of a 10 per cent solution of trichloroacetic acid. The mixture is shaken continually while the blood is being added. It is allowed to stand for 30 minutes and then filtered. 3 ml. of the filtrate are then transferred to a test-tube to which 1 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid is added. The mixture is allowed to stand at room temperature for at least 10 minutes and is then extracted with 4 ml. of ethyl acetate. The mixing of the two layers is effected by a capillary pipette through which a slow current of nitrogen is blown. After separation of the two layers, the lower one is removed by means of the same capillary pipette and transferred to another test-tube. This fraction is now extracted with 2 ml. of ethyl acetate and the ethyl acetate layer is then added to the tube containing the original extract. The extraction is repeated with another 2 ml. of ethyl acetate until the lower layer is completely colorless. The upper layer is then added to the other ethyl acetate extracts. Exactly 2 ml. of sodium carbonate are added to the combined ethyl acetate and mixed for 5 minutes by means of a slow current of nitrogen blown through the capillary pipette. At the end of this period, the sodium carbonate layer is transferred to another test-tube. This extraction is repeated twice more with the same amount of sodium carbonate. The combined sodium carbonate fractions are then extracted with 1 ml. of ethyl acetate and the sodium carbonate extract transferred to a special tube designed for use in the Evelyn colorimeter. 4 ml. of 2 N sodium hydroxide are added to this fraction, and the mixture well shaken. After 10 minutes the intensity of color due to the phenylhydrazone of pyruvic acid is determined in the photoelectric colorimeter with Filter 520.

The exact amount of pyruvic acid in a given sample is determined by the use of a standard curve. The pyruvic acid used in obtaining this curve is prepared immediately before use, by three

redistillations, the fraction boiling at 55–60° and 10 mm. of Hg being used. To obtain the actual pyruvic acid content of the blood in mg. per cent, the appropriate correction is made for the volume of sodium iodoacetate solution originally used.

### *Results*

1. The content of pyruvic acid in the blood has been determined in 60 normal subjects without evidence of physical or mental illness. The ages ranged from 8 to 48 years, twenty-four of our subjects being children under the age of 15. All samples were obtained on fasting subjects at rest in bed, and all determinations were made in duplicate. The figures varied from 0.77 to 1.16 mg. per cent, the average being 0.98 mg. per cent and the standard deviation  $\pm 0.09$ . Lu (6) has previously reported as normal values ranging from 0.4 to 0.75 mg. per cent. It should, however, be noted that she considered cases of treated vitamin B<sub>1</sub> deficiency as normal. Her subjects received large doses of vitamin B<sub>1</sub>, which in itself may account for the low figures obtained. Furthermore, her method does not allow for complete stabilization of pyruvic acid. The disappearance prior to precipitation (without the use of a stabilizing medium) is therefore reflected in the lower values obtained.

2. The addition of sodium cyanide to blood samples produces a greater disappearance of pyruvic acid than would occur in a similar length of time without the addition of this substance. Furthermore, this disappearance is not prevented by the presence of iodoacetate (Table III).

3. The addition of fluoride does not prevent the disappearance of pyruvic acid from blood samples standing at room temperature. If, however, fluoride and sodium iodoacetate are added together, the disappearance of pyruvic acid from such blood samples promptly stops.

4. In contrast to our findings in the blood, the pyruvic acid content of the cerebrospinal fluid remains constant even if the sample is allowed to stand at room temperature for 1 hour. These findings, together with the relationship of blood to spinal fluid pyruvate, will be discussed in a subsequent paper.

These results take on added interest in view of recent work on cocarboxylase. It has been demonstrated that the activation



of cocarboxylase by hexosediphosphate is inhibited by iodoacetate, but not by NaF (Lipschitz, Potter, and Elvehjem (7); Ochoa and Peters (8)). Furthermore Tauber (9) reported the activation of cocarboxylase by NaCN. In addition, Goodhart and Sinclair (10) have shown that cocarboxylase is present in the blood cells but not in the plasma or the normal cerebrospinal fluid. This

TABLE III

*Effect of Sodium Cyanide on Pyruvic Acid Content of Standing Blood*  
The results are expressed in mg. per cent of pyruvic acid.

Blood sample No.	With sodium iodoacetate	Without sodium moniodoacetate	
	Pptd. after 90 sec.	Pptd. after 15 min.	0.5 per cent NaCN; pptd. after 15 min.
1	1.05	0.48	0.22
2	0.85	0.19	0.05
With sodium moniodoacetate			
3	Pptd. after 2 min.	0.5 per cent NaCN;* pptd. after 2 min.	0.5 per cent NaCN;† pptd. after 30 min.
	1.06	1.04	0.34

\* NaCN added immediately before precipitation.

† NaCN added 28 minutes before precipitation.

fact may account for the stability of pyruvic acid in the cerebrospinal fluid which ordinarily contains practically no cells.

## SUMMARY

1. Moniodoacetic acid prevents the disappearance of pyruvic acid from non-precipitated blood.

2. A method is described for determining pyruvic acid in the blood.

3. Figures for the pyruvic acid content of blood, obtained on 60 normal subjects, varied from 0.77 to 1.16 mg. per cent.

4. Incidental data related to the effect of fluoride and sodium cyanide are reported.

5. The stability of pyruvic acid in the spinal fluid is reported.

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## REMOVAL OF THE IMPURITIES, NUCLEIC ACID AND POLYSACCHARIDE, FROM TUBERCULIN PROTEIN\*

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By the methods most generally used in the past for the purification of the tuberculin protein nucleic acid and polysaccharide often have not been removed. While in most of the final fractions the nucleic acid content varied from 4 to 8 per cent, in a few cases it was as high as 30 to 40 per cent and in others as low as 0.8 per cent. Such a low figure as the latter, however, could not be attained at will, and seemed to be dependent upon a low initial nucleic acid concentration in the source material. The polysaccharide content ranged from 3 to 40 per cent. However, the size of the tuberculin reaction produced in many series of tests in man or experimental animals by these preparations was independent of the quantities of nucleic acid and polysaccharide present. Therefore, both of these substances may be considered impurities and should be eliminated, if possible, in the purification of the protein, especially since the presence of the nucleic acid, in particular, modified many of the physicochemical properties of the protein, such as isoelectric point, electrophoretic mobilities, and solubilities.

### *Methods*

Nitrogen was determined by a modification of the micro-Kjeldahl method of Pregl, and the protein was calculated upon the basis of 16.3 per cent nitrogen previously determined (1) on the dry weight of a highly purified tuberculin protein.

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Nucleic acid was determined quantitatively by means of the Dische diphenylamine reaction (2). The blue color was measured in the Evelyn photocolormeter, with a filter of wave-length  $\lambda$  600, and the values obtained were found to satisfy Beer's law. The standard curve was made with a very pure thymus nucleic acid kindly given to me by Dr. Einar Hammarsten. Since many of the tuberculin preparations analyzed were highly colored, the corrections for this color were especially important. The following procedure was found to yield the most nearly correct results.

In two Pyrex tubes, 20 cm. long, were placed 3 cc. of water, and in two others 3 cc. containing an amount of the unknown solution which would be estimated to contain 0.02 to 0.5 mg. of nucleic acid. To one tube of each set were added 6 cc. of the blank reagent, made by adding 2.75 cc. of concentrated sulfuric acid (nitrogen-free, reagent grade) to 100 cc. of glacial acetic acid. To each of the remaining two tubes were added 6 cc. of the diphenylamine reagent, made by dissolving 1 gm. of diphenylamine in 100 cc. of glacial acetic acid plus 2.75 cc. of the concentrated sulfuric acid mentioned above. Both series of tubes were covered with glass covers and simultaneously immersed in a vigorously boiling water bath for exactly 10 minutes and then simultaneously removed and immersed in cold water.

The blank tube containing only water and the diphenylamine reagent was read against the blank tube containing water and the reagent without diphenylamine. This galvanometer reading, which is the blank on the reagents, should be about 96 to 99, and the amount of nucleic acid corresponding to this reading was considered to be the blank on the reagents. The tube containing the unknown solution was then read against its corresponding blank, which was the tube containing the unknown but no diphenylamine in the reagent. The concentration of nucleic acid corresponding to this galvanometer reading was determined from a standard curve obtained as described below, and from it was subtracted the blank.

The standard curve was obtained by plotting the amount of nucleic acid used against galvanometer values obtained by reading a series of tubes containing amounts of the pure Hammarsten nucleic acid, varying from 0.02 to 0.5 mg., against a blank containing only the reagent. This reaction was highly specific for

thymus nucleic acid and no color was obtained with the tuberculin polysaccharide or yeast nucleic acid.

Polysaccharide was determined by means of the carbazole reaction (2), and the color was also measured in the Evelyn photocolormeter, with a filter of wave-length  $\lambda$  540. Since this reagent will give a red color with the carbohydrate in both the tuberculin polysaccharide and in the nucleic acid, the result includes both of them. While a slightly higher value would be obtained for the nucleic acid carbohydrate if a filter of wave-length  $\lambda$  520 were to be used, one of wave-length  $\lambda$  540 gives the most nearly correct result in unknown mixtures of the two and was, therefore, used.

The brown color, formed so readily in these carbohydrate solutions when they are heated with the reagent which contains nearly concentrated sulfuric acid, absorbs chiefly at a wave-length of  $\lambda$  420, and adequate correction must be made for it if accurate results are desired. In addition to this correction, another is necessary for the blank reagents. The following procedure gives the most reliable results.

To each of two Pyrex tubes were added 9 cc. of the sulfuric acid reagent, consisting of 8 parts of concentrated sulfuric acid (nitrogen-free, reagent grade) and 1 part of water. The tubes were chilled in ice water and then to each was added 1 cc. of the unknown solution which was estimated to contain 0.02 to 0.25 mg. of carbohydrate, following which the tubes were again cooled. To one of the tubes was added 0.3 cc. of the alcoholic 0.5 per cent carbazole reagent, and to the other 0.3 cc. of alcohol alone. Both tubes were heated simultaneously in a boiling water bath for exactly 10 minutes and then chilled. The blanks, one consisting of water, sulfuric acid reagent, and the carbazole reagent, and the other of water, sulfuric acid, and alcohol, were heated at the same time.

Readings were made in the photocolormeter of carbazole blank against water blank, and then the unknown solution against its blank. The galvanometer readings were related to concentration of carbohydrate by means of the standard curve. The value for the reagent blank was subtracted from the value obtained on the solution. With sufficiently pure reagents a galvanometer reading of 90 or more should be obtained on the reagent blank.

The standard curve was obtained by plotting the galvanometer

values found for varying concentrations (0.03 to 0.3 mg.) of pure tuberculin polysaccharide (isolated a number of years ago and recently studied (1)) when read against their corresponding blanks.

Intracutaneous tests to determine potency were made in tuberculous guinea pigs and man, and the reactions were measured in three dimensions 24 and 48 hours after the injection.

#### EXPERIMENTAL

Experiments made by means of the Tiselius electrophoresis apparatus (3, 1) on highly purified, as well as on crude tuberculin protein fractions, were helpful in understanding the nature of the combinations between either the nucleic acid or the polysaccharide and the protein and thus in guiding their separation. For example, most preparations, no matter how extensive the chemical purification, showed in electrophoresis the presence of a main component, which proved to be the protein, and also usually two other substances. One of these was a very weak component, had a much higher mobility than the protein, and proved to be nucleic acid when isolated and tested with the diphenylamine reagent (2). The other, also a weak component, had practically no mobility and when separated by means of the compensation device of Tiselius and tested with the carbazole reagent (2) proved to be a polysaccharide.

Protein preparations, showing the presence of these two contaminants, were obtained when a great variety of different methods of purification had been used. For example, repeated precipitation with 2 or 10 per cent trichloroacetic acid, repeated precipitation with ammonium sulfate at the isoelectric point, pH 4.8, isoelectric precipitation with acetate buffer salts, electrodialysis, and dialysis at acid reaction, all gave preparations with a high nucleic acid and polysaccharide content.

One such product, PPD-12, which contained 9 per cent nucleic acid and 14.2 per cent polysaccharide, was used for a careful study of its mobility curve (Fig. 1) in the Tiselius electrophoresis apparatus. In all experiments the ionic strength of the solution was kept constant at 0.02 and citrate, acetate, or phosphate buffers were used, depending upon the pH desired. The current passing was always 3 to 4 milliamperes, and the concentration of the solutions averaged about 1 per cent. Since the boundary

anomalies are less pronounced for the descending than for the ascending boundaries (4),<sup>1</sup> the mobilities of the former boundaries were used in the graph in all cases. The abscissa indicates pH and the units on the ordinate represent the mobilities  $\times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$  of the descending boundaries. Since the PPD-12 was insoluble between pH 4.8 and 2.4, it was impossible to obtain an accurate isoelectric point, which was probably between pH 3 and 4.

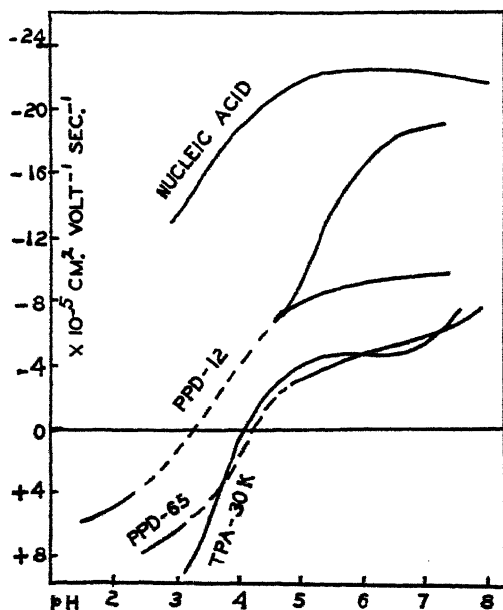


Fig. 1. Mobility curves. Mobilities are plotted against pH;  $\mu = 0.02$ . Phosphate buffer from pH 8 to 5.6, acetate buffer from pH 5.6 to 4.3, and citrate buffer from pH 4.3 to 1.5 were used. The dotted lines indicate the range of insolubility of the protein fractions.

The significant fact disclosed by the curve is that a highly mobile component splits off above pH 5.0, although at pH 5.0 as well as at pH 2.2 the fraction traveled as a single component. This would indicate either that at certain pH values the electrical properties were similar, or that a dissociation occurred at pH

<sup>1</sup> Tiselius, A., personal communication.



5.0, releasing the fast moving component, which, when isolated in the separation cell, proved to be largely nucleic acid. Such an association could occur between the imino group of histidine in the protein and the secondary phosphoric acid group of the nucleic acid, both of which dissociate in the region of pH 5 to 6. Therefore, the link, if there is one, between the protein and the nucleic acid must be a fairly loose combination which can be broken during electrophoresis in a properly chosen buffer.

Fig. 1 also shows the mobility curve for pure thymus nucleic acid (Hammarsten) run under identical conditions. The mobility curves, obtained under similar conditions, of the antigenic tuberculin protein molecule, TPA-30K (1) of molecular weight 35,000, which contains no nucleic acid, and of a non-antigenic protein molecule, PPD-65, of about 17,000 molecular weight, which contains only about 0.8 per cent nucleic acid, are practically identical, especially between pH 3.7 and 8.0. Both, however, had a lower mobility than the protein component of PPD-12, and of numerous other preparations containing nucleic acid that were studied. As a rule, the lower the content of nucleic acid in a preparation the nearer the mobility of the protein component approached that of the pure protein.

Furthermore, the mobility of the fast component in the PPD-12 was less than that of pure nucleic acid alone. Thus there appears to be some influence of each of the components on the mobility of the other. A similar observation has recently been made by Stenhagen and Teorell (5).

At all pH values the immobile polysaccharide was present as a separate component, indicating that it is not an integral part of the protein molecule. There seemed to be practically no influence of the presence of the polysaccharide on the properties of the protein, as there was in the case of the nucleic acid, and, therefore, no evidence for a combination between protein and polysaccharide. The content of polysaccharide merely decreased with each successive purification, a fact suggesting that previously it had been adsorbed.

The facts obtained by means of these mobility curve determinations indicated that it should be possible to remove these impurities from the protein by repeated electrophoresis. Therefore, 10 cc. of a 3.5 per cent solution of PPD-72-2, containing 11 per cent

nucleic acid and 13.5 per cent polysaccharide, were subjected to electrophoresis at pH 7.4, where it is evident from Fig. 1 that a maximum separation of the protein and nucleic acid will occur. All experiments were run at this pH in phosphate buffer,  $\mu = 0.1$ . Since the desired fraction, the protein, had a mobility between that of the two impurities, the nucleic acid and the polysaccharide, which it was hoped to remove, the separation was made in a somewhat different manner than is usually followed.

It was reasoned that if the highly mobile nucleic acid were allowed to travel as fast as possible toward the anode, and at the same time the compensation device were used to keep the protein component from leaving its original cells, the immobile polysaccharide would simultaneously be pushed toward the cathode. The two impurities could thus be removed and the remaining solution would have the original volume, but would contain less nucleic acid and less polysaccharide. It was then re-run in the same way, a procedure again permitting the removal of more nucleic acid and more polysaccharide. The process was repeated four times, as shown in Table I, yielding finally a protein solution with less than 1 per cent nucleic acid and 1.7 per cent polysaccharide by analyses. Electrophoretic diagrams (Fig. 2), obtained by means of the refractive index scale method of Lamm (6), showed the presence of a small amount of the faster component during the second run (*A* and *B*), and the absence of this component during the fourth run (*C* and *D*). The total concentration naturally decreased with each run and therefore no more than four such purifications were profitable in this experiment. The maximum degree of separation possible at any one run was limited by the height of the cell, since, as soon as the descending nucleic acid or polysaccharide boundaries reached the bottom cell, convection currents made further electrophoresis inadvisable.

It is of interest that the potency of the tuberculin protein was not decreased by the repeated purifications of the PPD-72-2 by electrophoresis, as shown in Table I. The average size reactions in eight tuberculous guinea pigs to comparable 0.005 mg. doses, based on the nitrogen contents of the different fractions, also showed no decrease in potency over the original solution.

This experiment demonstrates the possibility of separating both nucleic acid and polysaccharide from the protein in a more re-

liable manner than any of the numerous chemical procedures previously used. Furthermore, it shows that a large amount of the protein exists free of the nucleic acid or so loosely bound that

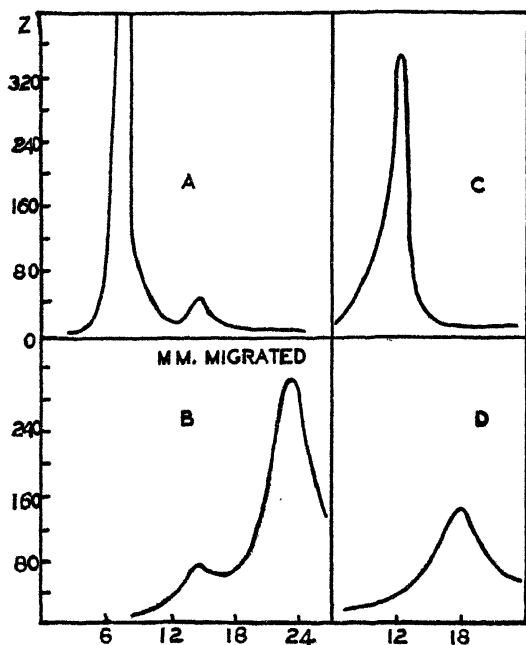


FIG. 2. Electrophoretic diagrams of PPD-72-2, scale method. The displacement of the scale lines,  $Z$ , in  $\mu$ , is plotted against the position of the displaced lines in mm. *A*, ascending boundary, second run, 35 minutes after the current was started, potential gradient,  $F = 5.90$  volts per cm., concentration 1.9 per cent, migration of protein =  $-9.4 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup>, of nucleic acid =  $-18.3 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> *B*, descending boundary, second run, migration of protein =  $-6.6 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> *C*, ascending boundary, fourth run, 1 hour after the current was started,  $F = 5.84$  volts per cm., concentration 1.06 per cent, migration of protein =  $-7 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> *D*, descending boundary, fourth run, migration of protein =  $-6.8 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup>

the two are easily separable, and indicates that the reason why previous methods used for isolating the protein have failed to release the nucleic acid is that the separations practically always took place at acid reactions, where the two substances have similar physicochemical properties or precipitate together.

TABLE I  
*Purification by Means of Electrophoresis*

PPD-72-2	Concentration of solution	Per cent of nucleic acid	Per cent of polysaccharide	Mobilities $\times 10^4$ cm. <sup>2</sup> volt <sup>-1</sup> sec. <sup>-1</sup> *		Average size skin reaction in 8 tuberculous guinea pigs	
				Fast component	Slow component	24 hrs.	48 hrs.
	<i>per cent</i>				<i>mm.</i>		<i>mm.</i>
Original solution.....	3.50	10.98	13.5	-10.3 (Not separated)		$16 \times 16 \times 2.4$	$17 \times 17 \times 2.7$
After 1st run.....	1.91	6.3	10.3				
" 2nd ".....	1.44	2.9	5.9	-17.6	-6.6		
" 3rd ".....	1.06	0.64	4.2	-16.0	-6.5	$17 \times 17 \times 2.4$	$16 \times 16 \times 2.7$
" 4th ".....	0.69	0.60	1.7	Absent	-6.8		

**\* Descending boundaries.**

TABLE II  
*Analyses and Potency of Purified Fractions*

PPD	Protein  mg. per cc.	Nucleic acid  mg. per cc.	Poly- saccharide  mg. per cc.	Skin tests in human beings					
				First dose, 0.01 $\gamma$			Second dose, 2.5 $\gamma$		
				No. tested	No. positive	Average size reaction  mm.	No. tested	No. positive	Average size reaction  mm.
PPD-IIIa.....	25.8	12.3	13.6						
PPD-IIIb.....	13.1	6.4	6.7						
PPD-IIIa2.....	10.8	0.09	0.8	41	25	$16 \times 18 \times 1.9$	13	10	$18 \times 19 \times 2.3$
PPD, standard.....				41	25	$15 \times 17 \times 1.9$	13	10	$17 \times 19 \times 2.3$

The obvious practical conclusion to be drawn from these results is that if the precipitations were to take place on the alkaline side of pH 5.0, at, for example, pH 7 or 8, it should be possible to make a much better separation.

Consequently, the following experiments were undertaken. Partially purified tuberculin was used which had been made from cultures of a human type tubercle bacillus grown on Long's synthetic medium for 8 weeks, heated in the Arnold sterilizer for 3 hours, filtered free of bacilli, and then concentrated and washed on the ultrafilter (11 per cent guncotton-glacial acetic acid membranes) with weak phosphate buffer of pH 7.3. The entire process after the Arnold sterilization was carried out at ice box temperature. Aliquot portions of this were purified in two different ways. In one case about 200 cc. were precipitated by 2 per cent trichloroacetic acid five times, being dissolved with as little sodium hydroxide as possible following each precipitation. The final precipitate was dissolved and washed on the ultrafilter free of sodium trichloroacetate with weak phosphate buffer, pH 7.3. The resultant solution was filtered for sterility on the Seitz filter and then preserved in the dry form by the cryochem process (7). This was called PPD-IIIa.

In the second case 50 cc. of the same original solution were precipitated by the addition of an equal amount of saturated ammonium sulfate previously neutralized with sodium hydroxide. Recently it has proved more satisfactory to neutralize the ammonium sulfate with solid disodium phosphate. This precipitation was carried out three times, at the end of which the supernatant fluid was water-clear and colorless. Solution of the precipitates was caused by weak phosphate buffer, pH 7.3. The final precipitate was dissolved and washed free from ammonium sulfate on the ultrafilter by means of weak phosphate buffer, pH 7.3, and then preserved in the dry form by the cryochem process (7). This was called PPD-IIIa2.

Table II shows the analyses on these fractions. A third fraction, PPD-IIIb, made from a similar original solution and in the same way as the PPD-IIIa, *i.e.* by means of 2 per cent trichloroacetic acid precipitation, is also included in Table II. It is clear from the results that the separation made at pH 7 to 8 yielded a product with about 0.8 per cent nucleic acid, as compared with

about 24 per cent nucleic acid when the precipitation was made with trichloroacetic acid. Likewise the polysaccharide content in the former case was about 7 per cent of the total product, as compared with 26 per cent for the latter.

The potency of this product in tests on both tuberculous guinea pigs and human beings in comparison with a standard PPD proved to be good. Table II shows the average size of the reaction in a sufficiently large series of tests on human beings to make the comparison reliable (8).

Products as free as this of nucleic acid can safely be standardized on the basis of their nitrogen content, since the pure tuberculin protein has been found (1) to contain 16.3 per cent nitrogen, based on the dry weight.

#### SUMMARY

Tuberculin potency determined by the skin reaction was dependent upon tuberculin protein only. It was independent of the nucleic acid and polysaccharide contents of tuberculin preparations, and, therefore, these impurities should be removed.

Nucleic acid and polysaccharide could not be removed quantitatively from the protein by isoelectric precipitation, by electro-dialysis, by dialysis at pH 2.1, by repeated precipitation by half saturation with ammonium sulfate at the isoelectric point, or by repeated precipitation with 2 or 10 per cent trichloroacetic acid.

Electrophoretic mobility curves revealed the fact that on the acid side of pH 5.0 the nucleic acid and protein migrated as a single component, whereas at less acid reactions than pH 5.0, the two traveled with very different mobilities. The mobility of each appeared to be influenced somewhat by the presence of the other. The polysaccharide was immobile at all pH values and there appeared to be no combination with the protein, except by adsorption. Therefore, by means of repeated electrophoresis at pH 7.3, it was possible to remove both nucleic acid and polysaccharide from the protein, with no loss in potency.

It was, furthermore, possible to remove both of these impurities from the protein by repeated precipitation on the alkaline side of pH 5.0, as by half saturation with ammonium sulfate at pH 7.0, with no loss in potency.

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## ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY

### IX. THE ELECTRIC MOBILITIES OF QUARTZ AND COLLODION PARTICLES IN MIXTURES OF HORSE SERUM AND SERUM PROTEINS IN RELATION TO THE MECHANISM OF FILM FORMATION

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In a recent communication, Moyer and Moyer (1) have shown that particles of quartz and collodion attain different electrophoretic mobilities after being exposed to dilute horse serum. The electrophoretic mobility-pH curve for the collodion particles in serum agreed completely with the curve obtained for particles in solutions of the serum globulin fraction. Comparison of the behavior of the quartz particles with the electric mobility curves of the two albumin constituents, A and B, isolated by the method of Kekwick (2), showed that the quartz when in serum becomes coated with a film resembling albumin A. The conclusions of Kekwick with regard to the identity of the electrophoretic behavior of the dissolved albumins A and B were substantiated by the measurements on the adsorbed albumins but only over the narrow pH range near the isoelectric point which he investigated (pH 4.0 to 5.5). At either end of this range, the mobility of adsorbed albumins A and B was different, with albumin A moving more slowly than B (1). By comparing their data with those of Tiselius (3) for the electrophoresis of the protein constituents in diluted serum, Moyer and Moyer concluded that albumin A may be nearly the same as the albumin Tiselius has isolated electrophoretically, while the coating on the collodion agreed most closely in behavior with his globulin  $\alpha$ . Rabbit and human serum were also investigated with similar results.

Although these investigations show that the films adsorbed on these particles are different, they do not give much information



about the mechanisms involved in their formation. This point can best be investigated by following the behavior of particles in mixtures of serum constituents with each other and with serum. In a similar investigation, using mixtures of gelatin with casein and egg albumin, Moyer and Moyer (4) have found that the resultant film depends on the proteins involved and, to a certain extent, on the nature of the particle. Two chief modes of approach were used: (1) coating the particle with one protein and then exposing it to the other, and (2) putting the particles into a mixture of the two proteins. The microscopic method of electrophoresis was used to determine the nature of the surface films formed on the particles. Their results, together with those in the literature, indicate that one protein may replace another at a surface, two proteins may be adsorbed side by side, or one may or may not be able to coat the other.

To discuss the various factors involved in film formation on particles when immersed in protein solutions, let us consider the case in which a particle already coated with one protein is placed in a solution of another protein. If enough more free energy is dissipated in film formation by the initial than by the second protein, no replacement of the first protein by the second can occur, in spite of the high concentration of the other protein. It is still possible, however, for the particle to become coated with the second protein by the formation of a double or multiple film. The formation of a double coat would be analogous to interaction between the same two molecules in the solution itself. It is unlikely that the anchored molecule will interact much more readily with a molecule of the second protein than would a free molecule unless it is altered upon adsorption to such an extent that new groups are made available (denaturation and unfolding). To determine definitely whether such profound changes have occurred might require, for any particular case, information from several different sources: chemical, physicochemical (electrophoresis, heat of adsorption, etc.), and immunological. Tentatively, however, we suggest the incomplete criterion that if the electric mobility of the film-coated particles is the same as that of the free molecules and, furthermore, if the protein on the particles is one which does not denature rapidly at the air-water interface at room temperature, no important changes in the

molecule should be expected upon adsorption. Quantitative information concerning the latter restriction, although desirable, is not available at present.

Correspondingly, the possibility that the anchored molecule will interact less readily than the free molecule with another protein cannot be entirely dismissed when specific groups, or perhaps a single group on each protein, are responsible for the interaction between the two proteins. For it may happen that the same specific group is preferentially attached to the surface of the particle and thus becomes unavailable. On the other hand, the failure of the second protein to replace the first in the time allowed does not necessarily warrant the conclusion that the free energy relationships are unfavorable, for, if the adsorbed protein molecules exchange very slowly with those in the solution, it might be necessary to wait a very long time for the system to come to equilibrium. Finally, if the second protein replaces the first, it can be concluded that the free energy changes involved in film formation by the respective proteins are of about the same order of magnitude or that they are greater (negative sense) for the second than for the first protein.

When uncoated particles are added to a mixture of two or more proteins, the situation is different, for there is now a chance for competition between the proteins in their relative rates of film formation. In this instance, the final result, if the various equilibria are not rapidly established, will depend mainly upon the relative rates of film formation for the two proteins rather than upon the free energy changes.

The same procedures as were followed by Moyer and Moyer have been used with the serum proteins and serum in the present investigation. In this paper, the attempt will be made to clarify the mode of film formation and the nature of the interaction between serum proteins and "inert" particles in relation to the biological aspects of the problem.

### *Methods*

The serum samples and protein preparations were the same as were used before (1). The albumin constituents, A and B, had been prepared by the method of Kekwick (2). All measurements have been made in phosphate buffers, pH 7.6, at an ionic strength

of 0.1. The protein concentrations used were as follows: albumin A, 0.1 per cent; albumin B, 0.1 per cent; globulin, 0.3 per cent. A higher concentration was used for the globulin because of its higher molecular weight and also because it does not represent a single component but is a mixture of at least three globulins of which one seems to be selectively adsorbed (1). The term "globulin" will be used in this communication to refer to this constituent of the globulin fraction. If lower concentrations of these globulins were used, results were not so uniform. The experiments were performed by exposing quartz or collodion to strong solutions of the proteins (greater than 3 per cent) for an hour or more and then, after addition of the buffer constituents, the second protein or serum was added and the final volume of the solution was allowed to stand at room temperature for at least another hour before measurements were made. In the experiments on mixtures, the stock protein solutions were mixed and the particles were added to them and allowed to stand for about an hour; whereupon the mixture was diluted to the proper concentration by the addition of the salts and water and again allowed to stand for at least an hour. These time intervals were found sufficient to produce uniform results. Electrophoresis and pH measurements were made by our usual techniques (1, 5).

It might have been desirable to make measurements at more than one pH value but, since the amounts of protein were limited and the electrophoresis curves of Moyer and Moyer (1) for horse serum and its proteins showed no discontinuities in the coating at different pH values, these were not carried out.

#### EXPERIMENTAL

Table I shows typical results obtained with binary mixtures of these three protein fractions at 25°. The experiments marked "Control" were performed without the second protein and represent mean mobilities taken from the pH-mobility curves of these constituents at this pH. By comparison of the experimental mobilities with those of the controls, the nature of the resultant surface, or at least its outer coating, can be determined.

In Experiment 1, both quartz and collodion were initially coated by albumin B but the outer surface changed to a film of albumin A only on the quartz. It is highly probable that this change on

the quartz is the result of albumin A replacing B and not the adsorption of albumin A on B. For, if albumin A tended to be adsorbed by albumin B on quartz, there is little reason to believe that it would not have been adsorbed by the film of albumin B on the collodion. It seems that if albumin B is once on collodion, albumin A cannot replace it or be adsorbed by it. There is, of course, the possibility that albumin B is altered in its capacity to

TABLE I

*Electrophoretic Mobilities of Quartz and Collodion Particles after Treatment with Serum Proteins*

pH 7.6;  $\mu = 0.1$ .

Experiment No.	1st protein	2nd protein	On quartz		On collodion	
			Mobility	Resultant surface	Mobility	Resultant surface
			$\mu$ per sec.		$\mu$ per sec.	
Control	Albumin A		1.12	Albumin A	1.12	Albumin A
"	" B		1.32	" B	1.32	" B
"	Globulin		0.76	Globulin	0.76	Globulin
1	Albumin B	Albumin A	0.99	Albumin A	1.36	Albumin B
2	" A	" B	1.03	" "	1.16	" A
3	Mixture of A and B		1.08	" "	1.39	" B
4	Albumin B	Globulin	0.95	Both	0.70	Globulin
5	Globulin	Albumin B	0.74	Globulin	0.67	"
6	Mixture of B and globulin		0.85	"	0.74	"
7	Albumin A	Globulin	1.02	Albumin A	0.66	"
8	Globulin	Albumin A	0.76	Globulin	0.72	"
9	Mixture of A and globulin		0.77	"	0.79	"

adsorb A after adsorption by quartz or collodion but, if this is the case, it is not accompanied by a detectable change in its electric mobility. Let us assume for the present that replacement has occurred and discuss later the possibility of this other, less likely, change in the protein as the mechanism involved.

When quartz or collodion is first coated with albumin A (Experiment 2), albumin B is not adsorbed. In the mixture (Experiment

3), albumin B is evidently more rapidly adsorbed on the collodion than A, so that the A cannot go on, for, if albumin A had been adsorbed first, then B would not have been adsorbed (*cf.* Experiment 2) and the final surface would be albumin A. Evidently once albumin B is on the collodion, albumin A is repelled. It is probable that this difference in the rates of film formation involves differences in the constitution of the proteins, for both have the same molar concentration and diffusion constant (2). Quartz becomes coated with albumin A in the protein mixture. This was expected, for Experiment 2 has shown that if albumin A reached the surface first, B would not be adsorbed and Experiment 1 indicates that if albumin B got there first A could replace it. Either mechanism would give albumin A as the sole coating. If our interpretation of the first part of Experiment 1 is correct (that albumin A replaces B on quartz), it can be concluded that albumin A is more strongly adsorbed by quartz than B; that is, that the free energy changes are in favor of the adsorption of albumin A. On the other hand, for the collodion surface, it can only be concluded that albumin B is more rapidly adsorbed than A. It is also indicated by Experiments 1 to 3 that both the serum albumins are more "irreversibly" adsorbed by collodion than by quartz.

Considering next Experiments 4 to 6 with quartz and collodion, we see that albumin B cannot replace globulin or be coated on it, whereas globulin can partially replace albumin B on quartz and completely replace it on collodion. According to the criteria that we have applied above, it is likely that these are partial and complete replacements respectively, rather than coating-over of globulin on albumin B. No interaction between albumin and globulin occurs in serum, for they migrate independently in an electric field (3). Nor is it likely that globulin would tend to coat over albumin B completely when it is anchored to collodion and incompletely when on quartz, for when covered by albumin B alone, both quartz and collodion have the same mobility and therefore their surface films must be nearly identical. If the interpretation that the globulin actually replaces albumin B on collodion is accepted, it follows that globulin is more strongly adsorbed than albumin B on collodion. In the case of quartz, it appears that the free energy of adsorption is not greatly different

for the two, although, of course, this is uncertain, for complete equilibrium was not attained. If complete equilibrium had been attained, the results in the first part of Experiments 4 to 6 would have been identical.

Almost completely similar results were obtained in Experiments 7 to 9. These can be interpreted in the same way as the results in Experiments 4 to 6 if albumin A is substituted for albumin B. There is one exception: globulin seems unable to replace or coat albumin A on quartz. These experiments lead to the conclusion that globulin is more rapidly adsorbed than albumin A on quartz and that the equilibria involved are slowly established.

If the replacements postulated above actually occur, their mechanisms must be more complicated than the simple one that might be assumed *a priori*: desorption of a molecule of the first protein followed by a competition between the molecules of the two proteins for the vacant space on the surface, for, of course, the desorption would be the slow step. If its rate were independent of the kind of neighboring molecules on the surface, when replacement of a given protein on a given surface is found to occur, it would be expected that such replacements of the same protein on the same surface would occur for all proteins which are more strongly adsorbed than the first protein on the given surface. Thus, since globulin can replace the albumins on collodion, then replacement of albumin B by A or albumin A by B, depending upon which is more strongly adsorbed, should also occur on collodion. Because these replacements do not occur, it must be concluded that if globulin actually replaces the albumins on collodion, there must be some mechanism by which it can increase the rate of desorption of the albumins. One possibility is that a globulin molecule can accelerate the desorption of a neighboring albumin molecule, and thus, after the creation of a few vacant spaces by ordinary desorption, the process would continually increase in rate (kind of autocatalysis).

It has been admitted above that some of these results could be interpreted by supposing a change to be produced in the protein by adsorption which could alter its capacity for adsorbing the dissolved protein. If we assume that albumin A is so altered on its adsorption by the collodion in Experiment 7 that it can adsorb globulin but is unaltered by the quartz, then we must assume in

Experiment 2 that it has not been altered enough to adsorb albumin B. In Experiment 4, if we assume that the albumin B on collodion is altered enough to permit it to adsorb globulin, then we must assume that it has not been changed enough so that it can adsorb albumin A in Experiment 1. If we assume that it is altered enough by quartz in Experiment 1 so that it can adsorb albumin A, then it must not have been changed enough to be able to adsorb globulin completely in Experiment 4. This alteration and double film hypothesis seems much more unlikely than replacement, especially when it is recalled that the electric mobility

TABLE II

*Electrophoretic Mobilities of Quartz and Collodion Particles Placed in Serum after Treatment with Albumin A or Globulin*

pH 7.6;  $\mu = 0.1$ .

1st coating	Serum dilution	On quartz		On collodion		Specific resistance	Remarks
		Mobility	Resultant surface	Mobility	Resultant surface		
		$\mu$ per sec.		$\mu$ per sec.		ohms	
Albumin A	3:50	0.93	Albumin A	0.80	Globulin	160	Control
	3:50	0.92	" "	0.73	"	160	
Albumin A	1:50	1.13	" "	0.80	"	188	Control
	1:50	1.12	" "	0.84	"	188	
Globulin	1:50	1.13	" "	0.80	"	188	Control
	1:50	0.83	Globulin	0.73	"	188	

of any one of these proteins on quartz or collodion is the same within the limits of error and in good agreement with the value for the dissolved protein (6-8). The possibility of these results being affected by an interaction of the protein in solution does not have much support, in view of the comparison by Tiselius (3) of the mobilities of mixed and isolated serum proteins.

In Table II a comparison of the behavior of quartz and collodion particles placed in serum with that of similar particles which had been previously coated with albumin A or globulin before the addition of serum is presented. The measurements made in the presence of serum at a dilution of 3:50 cannot be directly compared

with those made at a 1:50 dilution, for the higher concentration affects the ionic strength, as shown by the change in the specific resistance. Consequently each experiment must be compared with its proper control. The resultant surface has been indicated after each mobility value.

When serum itself is used as the second component, the results are essentially the same as in Table I. Albumin A persists on the quartz but appears to be replaced by serum globulin at a collodion surface. This result was found at both serum concentrations. Globulin, once on the surface of quartz, cannot be replaced by the albumin of the serum. It will be noticed that the results shown in Experiment 9 (Table I) do not agree with those of the controls for quartz placed in serum. This may be due to the differences in the protein concentrations in these experiments, for such differences should influence the rates of film formation. The rest of the data in Table II agree with those in Table I.<sup>1</sup>

Notice that globulin when coated on quartz appears either to be irreversibly adsorbed or the most strongly adsorbed of the three proteins, for in no case does replacement of globulin by albumin occur. On collodion, of course, globulin is more strongly adsorbed than albumin A or B. Finally, albumin A is more strongly adsorbed on quartz than albumin B. With the additional assumption that globulin can replace the albumins whenever the free energy differences are favorable, the tendency to be adsorbed on quartz is roughly in the order: albumin A > albumin B = globulin. On collodion, we can only say globulin > albumin A or B.

#### DISCUSSION

In the present experiments the nature of the particles frequently influences the composition of the resultant surface film. It has been generally found that hydrophilic substances are better adsorbed on hydrophilic surfaces, such as quartz or silica gel, and that hydrophobic substances are better adsorbed by hydrophobic surfaces such as carbon, collodion, or paraffin oil. The nature of the solvent can also influence the results. Berl and Wachen-

<sup>1</sup> A few experiments performed by measuring the mobilities of quartz and collodion in undiluted serum gave mobility values near  $0.4\mu$  per second, with the difference in mobility between the two particle types being less than the experimental error.



dorff (9) have found that aqueous solutions of crystal violet were better adsorbed by carbon than by silica gel, whereas this state of affairs was reversed when the dye was dissolved in tetralin. Holmes and McKelvey (10) have reported that Traube's rule, developed for the adsorption of dissolved substances in water by non-polar adsorbents, is reversed when a polar adsorbent, such as silica gel, is used and the fatty acids are dissolved in toluene. In the aqueous solution, the hydrocarbon ends are evidently turned toward the carbon, while in toluene the carboxyl groups are turned toward the silica gel. From an investigation of the adsorption of *cis*- and *trans*-azobenzenes, Freundlich and Heller (11) have drawn the following conclusions.

"The *cis* isomer is adsorbed more strongly by aluminum oxide, particularly in petroleum ether, less in methyl alcohol; the *trans* isomer is adsorbed more strongly by charcoal, particularly in methyl alcohol, less in petroleum ether. This is in agreement with other experimental results, according to which the more hydrophilic solute, here the *cis* isomer, is adsorbed more strongly by a hydrophilic adsorbent, here aluminum oxide, in a more hydrophobic medium, here petroleum ether, than by a more hydrophobic adsorbent, here charcoal, in a more hydrophilic medium, here methyl alcohol."

In applying these observations to the present case we shall assume that the serum albumins are relatively more hydrophilic than the globulins. This is a common assumption which is probably justified. Indeed, Spiegel-Adolf (12) finds that  $\text{Al}_2\text{O}_3$  adsorbs serum albumin better than pseudoglobulin, which is better adsorbed than euglobulin. In other words, the more hydrous albumin tends to be adsorbed better by this hydrophilic surface.<sup>2</sup>

<sup>2</sup> Dummett and Bowden (13) have attempted to show that the nature of the underlying surface affects the electric mobility of adsorbed oxyhemoglobin. As pointed out before (5), they made their measurements with a cylindrical cell, using 0.21 and 0.79 of the total depth as the stationary levels, instead of the correct positions at the level 0.147 from the top or floor of the cell (8). In addition, they appear to have neglected the important correction described by Henry (14) for the effect of the curvature on the apparent position of the stationary level. Unless this correction has been used, all measurements made in cylindrical microelectrophoresis cells are open to question. Hence it appears that Dummett and Bowden made their observations at a depth at which the particles were being

Fåhræus (17) states that the rate of sedimentation of red blood cells in plasma is markedly affected by changes in the globulin concentration. The globulins increase markedly in pregnancy, when the settling effect is most pronounced. Albumin is without influence on this phenomenon but it is markedly affected by globulins or fibrinogen, especially by the latter.

Linzenmeier (18) has found that the rate of settling is markedly inhibited by previous treatment of the plasma with bolus alba, kaolin, or carbon, whereas  $\text{Fe}_2\text{O}_3$ ,  $\text{Al}_2\text{O}_3$ , or  $\text{Ce}_2\text{O}_3$  had no effect. He ascribes this phenomenon to the removal of positively charged substances by the negative colloids. It seems more plausible, however, that the oxides were ineffective in the presence of plasma because of their hydration and not because of their positive charges (for  $\text{Al}_2\text{O}_3$  and  $\text{Fe}_2\text{O}_3$  will adsorb proteins on either side of their isoelectric points (16, 19)), while the carbon, etc., was able to adsorb other, relatively hydrophobic, negatively charged substances, such as globulins. Apparently the active substance is preferentially adsorbed by the more hydrophobic surfaces, while the other serum proteins are preferentially adsorbed by the more hydrophilic surfaces. The subject of red blood cell sedimentation has been recently investigated by Monaghan and White (20) by electrophoresis.

Jones (21) has shown that previous exposure of the presumably hydrophilic hog cholera bacillus to normal serum does not alter its reaction to its homologous antiserum, which, in the light of these results, probably involves protein replacement. It would be interesting to investigate the effects on immunological behavior of a protein capable of being irreversibly adsorbed, such as gelatin.

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affected by electroosmosis. It is incorrect to assume (15) that isoelectric point determinations made at levels other than the stationary levels will give correct values, unless the coatings of the cell and of the particle have identical  $\zeta$  potentials. They report that hemoglobin coated on carbon, copper, and quartz, from solutions at a concentration of 0.09015 per cent hemoglobin, exhibited wide differences in isoelectric point. It is not clear in all these cases that particles and wall were both completely coated by such concentrations. In any case, if they were, and the  $\zeta$  potentials of wall and particles were different, as their results imply, their isoelectric points are erroneous, for it is only when wall and particles have an identical surface constitution that measurements of the isoelectric point are independent of the depth (16).

Perhaps relatively hydrophobic bacteria, such as members of the acid-fast group (22), might exhibit a different behavior than that found by Jones.

Freund (23, 24) has investigated the toxicity of collodion particles which had been exposed to diphtheria and tetanus toxins, subsequent to or preceding their immersion in tetanus or diphtheria antitoxins. We present his results in Table III in a manner

TABLE III  
*Treatment of Collodion Particles with Toxins and Antitoxins*  
(Data of Freund (23, 24))

1st immersion	2nd immersion	Toxicity	Remarks
Tetanus toxin		Toxic	Control
Diphtheria toxin		"	"
Tetanus antitoxin	Tetanus toxin		Coating-over
" "	" " then tetanus antitoxin	Non-toxic	Neutralization and coating-over (?)
Diphtheria anti- toxin (or normal serum)	Tetanus toxin	"	No replacement or coating-over
Tetanus toxin	" antitoxin	"	Neutralization and coating-over (?)
" "	Diphtheria anti- toxin (or normal serum)	Toxic	No replacement or neutralization
Diphtheria anti- toxin	Diphtheria toxin	"	Coating-over
Tetanus antitoxin (or normal serum)	" "	Non-toxic	No replacement or coating-over
Diphtheria toxin	" anti- toxin	"	Neutralization and coating-over
" "	Tetanus antitoxin (or normal serum)	Toxic	No replacement or neutralization

similar to that used for the serum proteins given in Tables I and II. It will be seen at once that his data are often complicated by chemical interactions between the antigen and its antibody, leading to the formation of double films. Furthermore, it might be anticipated from the fact that antibodies are globulins that irreversible adsorption at the collodion surface would be encountered more often than replacement.

It is noteworthy that when the collodion is first coated with

protein from a solution of globulins containing only non-specific antibodies, the tetanus or diphtheria toxin cannot replace it or coat over it. Since it has been shown that globulin is strongly, and apparently irreversibly, adsorbed by collodion, this result is in accord with ours. When, however, the collodion is first coated from a solution of globulins containing a specific antibody and then treated with the corresponding toxin, the particle becomes toxic. Correspondingly, when the particles are first coated with toxin and then put into a solution of non-specific globulins, they remain toxic. Therefore, no replacement of toxin by globulin occurs. Freund (24) suggests the following explanations: (1) there are two groups on the antitoxin, a combining and a neutralizing group, of which the neutralizing group is held by the collodion; (2) it is possible that antitoxins on collodion adsorb more units of toxin than they can neutralize; (3) neutralization occurs only if antitoxin is on the outside. If such particles were heated to 55° for half an hour to destroy the toxin, antitoxin was released on injection into mice. This finding argues against the possibility of there being a replacement of toxin by antibody. Collodion treated with diphtheria toxin flocculated when treated with antitoxin, indicating that the antibody coats over the antigen.

It appears likely that in certain instances replacement may take place. Shaffer and Dingle (25) have found that a film of egg albumin can be deposited from 2 per cent solutions on a chromium slide covered with Y films of stearates (26). The thickness of the protein film was found to be 40 Å. by the Blodgett technique of comparing interference colors; this is approximately a monolayer. After addition of anti-egg albumin serum, an increase in thickness of 100 Å. was noticed. Immersion of this slide in the original egg albumin solution caused the film thickness to decrease to the original 40 Å. Whether the antigen-antibody complex is replaced by the egg albumin or whether the antibody globulin comes off, laying bare the original egg albumin film, cannot be decided from the data.<sup>3</sup> It is interesting that normal or heterologous horse or rabbit sera failed to adhere to the egg albumin films.

<sup>3</sup> If, for instance, the globulins make up part of the mosaic of the blood capillary wall, a possible mechanism of the whealing reaction to antigens in suitably sensitized individuals suggests itself: temporary increase in permeability caused by removal of the specific globulin antibody from the capillary membrane.

That protein exchange may take place within biological membranes is suggested by the recent work of Furchgott and Ponder.<sup>4</sup> These authors have found that the tendency of rabbit erythrocytes to become spherical when enclosed between slide and cover-slip may be inhibited by treating the cells with the carbohydrate-poor fraction of serum albumin (probably albumin B). Furthermore, they have shown that the "antisphering factor" (crystalbumin) is actually removed from the solution in amounts sufficient to form a monolayer on the cell surfaces. Yet no detectable change in the electric mobility of the cells occurs. Apparently then, according to these authors, the serum albumin must actually go into the membrane to become part of the mosaic, instead of being adsorbed on the surface of the cell.

#### SUMMARY

The electrophoretic mobilities of quartz and collodion particles were determined after exposure to mixtures of serum proteins or after they had been coated with one protein and then exposed to another or to serum. The results indicate that there is little tendency for the various constituents used to adsorb on each other, although one protein may replace another at a surface. The result is usually a film of one of the protein components rather than a mosaic. The nature of the underlying surface influences the adsorption, with the more hydrophilic proteins being adsorbed more readily by the more hydrophilic surface, and *vice versa*. In certain cases the results are complicated by irreversible adsorption. The biological significance of these findings is discussed.

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## CHEMICAL STUDIES ON THE PITUITARY "ANTAGONIST"

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There is present in extracts of the anterior pituitary a fraction which when given intraperitoneally antagonizes the effects produced by gonadotropic agents. At one time Evans *et al.* (1) claimed a separation of the "antagonist"<sup>1</sup> from their so called interstitial cell-stimulating hormone, but recently have stated that the assumption of a separate antagonist apart from the interstitial cell-stimulating hormone appears unjustified (2). Bunde and Hellbaum (3), however, have studied the stability of the "antagonist" at various pH levels and at 100°, and found that it is stable under conditions which destroy the gonadotropic fractions. The purpose of this study is to compare the "antagonist" with the gonadotropic substances previously studied in this laboratory (4-6) in regard to stability and reactivity to a wide range of chemical reactions, in an effort to establish a chemical difference. Since the response to gonadotropic extracts of pituitary origin is largely dependent upon the rate of resorption, as shown by subcutaneous injections (7), this factor must be considered in any interpretation of results. It was necessary in this study to establish the influence of delayed resorption or its equivalent, divided dosage, in the case of intraperitoneal injections. That this influence might be of the greatest importance was suggested by the contrary conclusions recently reached by two workers in the field.<sup>2</sup>

<sup>1</sup> The use of the terms antagonist, follicle-stimulating hormone, luteinizing hormone, and interstitial cell-stimulating hormone does not imply the acceptance of these concepts as scientifically established. They are used to connote effects produced by certain pituitary extracts under very arbitrary experimental conditions as described in the hormone literature.

<sup>2</sup> Fevold (8), comparing the response of rats to intraperitoneal injection



## EXPERIMENTAL

*Assay for Antagonist*—The pituitary gonadotropic preparation, which has been kept as a powder since 1935 and used in a number of studies (6, 7), was found to manifest the antagonist effect to a high degree and was used in the present studies. The measure of its effectiveness is given in Table I and serves as the basis for its assay. The gonadotropic effect observed when 1 mg. of the preparation is given subcutaneously is reduced by approximately 80 per cent when 1 mg. of the same preparation is simultaneously injected intraperitoneally. 0.25 mg. given intraperitoneally reduced the effect 50 per cent.

TABLE I  
*Assay of Standard Gonadotropic Powder for "Antagonist"*

Subcutaneous dose per rat*	Intraperitoneal dose per rat	Average ovarian weight at end of experiment†	No. of rats used
mg.	mg.	mg.	
1.0		111 ± 11	9
1.0	1.0	30 ± 3	8
1.0	0.5	39 ± 3	9
1.0	0.25	63 ± 9	6
0.5		50 ± 6	6
0.5	0.5	21 ± 1	6
	1.0	16 ± 1	6

\* 0.5 mg. of Cu as  $\text{CuSO}_4$  per dose per rat was added and the pH adjusted to 8.5.

† Mean plus standard deviation of the mean.

In the assay of the chemically treated preparations groups of 22 to 23 day-old female litter mate rats were used. All the rats received subcutaneously 1 mg. of hypophyseal preparation, augmented with 0.5 mg. of Cu as  $\text{CuSO}_4$ . A division of the group

with the response to subcutaneous injection, has concluded that his so called follicle-stimulating hormone is ineffective when injected into the peritoneal cavity, while his so called luteinizing hormone is as effective when injected intraperitoneally as when injected subcutaneously. Jensen *et al.* (2), however, report that their follicle-stimulating fraction is effective in normal or hypophysectomized rats, whether subcutaneous or intraperitoneal injections were made.

received in addition 1 mg. of the preparation intraperitoneally. Another division or other divisions of the group received in addition an equivalent of 1 mg. of the chemically treated preparation intraperitoneally. Copper was added only to the preparations given subcutaneously, its addition assuring delayed resorption and maximum response (7). By using six rats per preparation

TABLE II  
*Influence of Divided Dosage and Injection Route upon Ovarian Response*

Experi- ment No.	Total dose per rat	Injection route	No. of intra- peri- toneal injections per day	Ovarian weight	Uterine weight	No. of rats used
				mg.	mg.	
1	1 mg.	I.*	1	16	27	5
	1 "	"	5	28	63	5
	{ 1 "	"	1	25		5
	{ 1 " + 0.5 mg. Cu	S.				
	{ 1 " + 0.5 mg. Cu	I.*	5	55		5
2	1 "	S.				
	1 "	I.†	1	17	25	4
3	1 "	"	5	29	64	4
	0.5 " + 0.5 mg. Cu	S.		50		6
	{ 0.5 " + 0.5 " "	"		43		5
	{ 0.5 " + 0.5 mg. Cu	"		21		6
	{ 0.5 " + 0.5 mg. Cu	I.	1			
4	0.5 " + 0.5 mg. Cu	S.		62 ± 7		12
	{ 0.5 " + 0.5 " "	"		37 ± 3		12
	1 "	"				

I. = intraperitoneal; S. = subcutaneous.

\* In a concentration of 1 mg. in 1 cc.

† In a concentration of 1 mg. in 2 cc.

it was possible to detect the survival of 25 per cent of the antagonist. The destruction of less than 50 per cent of the antagonist was not measurable by the assay as performed. In all cases, dosage was performed once daily for 4 days, the animals being sacrificed the 5th day.

*Influence of Divided Dosage*—When the intraperitoneal dosage was made in five injections instead of one daily injection the

TABLE III

*Stability and Reactivity of Antagonist to Chemical Reactions*

Experiment No.	Treatment of material injected intraperitoneally	Mean ovarian weight increase as per cent of that produced by subcutaneous injection alone	No. of rats used
		<i>per cent</i>	
1	Untreated	19	6
	7 mg. powder, 2 cc. H <sub>2</sub> O, 200 mg. NaHCO <sub>3</sub> , 70 mg. (CH <sub>3</sub> CO) <sub>2</sub> O, 24 hrs.	126	6
2	Untreated	30	7
	10 mg. powder, 2 cc. H <sub>2</sub> O, 250 mg. NaHCO <sub>3</sub> , 70 mg. (CH <sub>3</sub> ) <sub>2</sub> SO <sub>4</sub> , 24 hrs.	103	7
	10 mg. powder, 2 cc. H <sub>2</sub> O, 250 mg. NaHCO <sub>3</sub> , 40 mg. $\beta$ -naphthoquinone sulfonate, 24 hrs.	84	7
3	Untreated	16	6
	20 mg. powder, 2 cc. H <sub>2</sub> O, 0.1 cc. CH <sub>3</sub> COOH, 0.25 cc. 0.1 N I, 3 hrs., then 0.25 cc. 0.1 N Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , then pH 6.0	32	6
4	Untreated	13	7
	100 mg. powder, 7 cc. H <sub>2</sub> O, 8 cc. buffer pH 5.8, 1 cc. 0.1 N I, 2 hrs., then pptn. with acetone	77	7
5	Untreated	27	7
	15 mg. powder, 7 cc. H <sub>2</sub> O, 100 mg. NaHCO <sub>3</sub> , 0.25 cc. 0.1 N I, 1 hr.	93	7
6	10 mg. powder, 2 cc. H <sub>2</sub> O, 30 mg. CH <sub>3</sub> COOH, 1 hr., then pH 6.0	52	7
	20 mg. powder, 4 cc. H <sub>2</sub> O, 60 mg. (CH <sub>3</sub> CO) <sub>2</sub> O, 1 hr., then alcoholic pptn.	77	6
	Untreated	20	7
7	20 mg. powder, 5 cc. H <sub>2</sub> O, pH 6.5, heated 2 hrs., at 98°	106	7
	Untreated	14	6
8	10 mg. powder, 2 cc. H <sub>2</sub> O, pH 6.5, heated 2 min. at 98°	77	6
	Untreated	23	6
9	10 mg. powder, 10 mg. cysteine HCl, 4 cc. H <sub>2</sub> O, pH 4.0, 1st dose after 1 hr.	38	6
	Untreated	20	4
10	30 mg. powder, 200 mg. NaHCO <sub>3</sub> , 60 mg. cysteine HCl, 10 cc. H <sub>2</sub> O under oil 48 hrs.	68	4
	Untreated	18	6
11	10 mg. powder, 5 cc. H <sub>2</sub> O, 200 mg. NaHCO <sub>3</sub> , 20 mg. diazobenzene sulfonate, 1 hr.	76	6
	Untreated	18	6

antagonist effect was reduced by 50 to 75 per cent (Experiment 1, Table II). Moreover, intraperitoneal injections alone when given in sufficient number produced considerable follicle stimulation, so that we are forced to disagree with Fevold (8) that the intraperitoneal route fails to produce follicle stimulation (see Experiments 1 and 2, Table II). The most illuminating effect is, however, suggested in Experiment 3 and demonstrated in Experiment 4, Table II, in which it is clearly shown that the antagonist effect may be produced by use of the subcutaneous injection route. The gonadotropic effect observed when 0.5 mg. of the preparation, augmented with copper to delay resorption, is given subcutaneously is reduced by approximately 50 per cent when 1 mg. of the same preparation without the addition of copper is simultaneously injected subcutaneously in another area.

*Chemical Reactions*—In Table III are recorded the effects by intraperitoneal injection of the chemically treated preparations compared in litter mates with the effects of the untreated preparation in reducing the increase in ovarian weight produced by subcutaneous injection alone; the increase produced by subcutaneous injection alone has arbitrarily been assigned a value of 100 per cent.

The results indicate that complete or nearly complete destruction of the antagonist was brought about in mildly alkaline solution by acetic anhydride, dimethyl sulfate,  $\beta$ -naphthoquinone sulfonate, iodine, and diazobenzene sulfonate. At pH 6.5, 2 hours heating of the solution at 98° resulted in complete inactivation. 2 minutes heating produced almost complete inactivation. Standing 1 hour in 1.5 per cent acetic acid solution produced considerable inactivation. The same concentration of acetic anhydride caused further inactivation. In acetic acid solution, iodine produced precipitation with little loss of activity. Cysteine in acid solution produced little inactivation. In mildly alkaline solution, in excess, and on prolonged treatment, considerable inactivation resulted.

#### DISCUSSION

In these studies, no difference in behavior between the antagonist and the gonadotropic pituitary preparation (4, 5) with respect to chemical reaction is noted. We previously reported that the pituitary preparation is more sensitive to methylation (5) than

the urinary or mare serum gonadotropic hormones, and Fraenkel-Conrat *et al.*<sup>3</sup> (9) have reported greater sensitivity of the former to prolonged cysteine treatment. There is, consequently, chemical evidence that urinary prolactin and the mare serum hormone are not identical with the so called luteinizing hormone, interstitial cell-stimulating hormone, or antagonist fractions. However, no difference in chemical reactivity for the so called luteinizing, follicle-stimulating, and antagonist fractions is thus far forthcoming.<sup>4</sup> The report of Bunde and Hellbaum that the antagonist survives 100° while the gonadotropic activity is destroyed was not confirmed by the behavior of our extract. Bunde and Hellbaum did not assure maximum gonadotropic response by delayed resorption in their assay and consequently complicated their whole study.

The protagonists for the multiple hormone theory have shown that it is possible to separate two fractions from the pituitary: one which under arbitrary conditions of assay will show follicle stimulation before interstitial cell stimulation; the other under the same arbitrary conditions of assay will show interstitial cell stimulation before follicle stimulation. The antagonistic effect is revealed in both fractions, depending on the assay procedure, and the luteinizing hormone effect has never been divorced from the follicle-stimulating fraction; even the advocates of the dual hormone theory have now abandoned luteinization as a point of differentiation.

Those opposed to the multiple hormone theory have shown that it is possible to produce follicle stimulation, luteinization, or antagonism at will with an unfractionated extract, depending upon the manipulation of dose and rate of resorption. They have also

<sup>3</sup> Since Fraenkel-Conrat *et al.* did not consider the alternate possibility that cysteine action might change the rate of resorption, their statement was not proved. We have repeated the experiment of Fraenkel-Conrat *et al.*, assaying the reaction products by procedures employing delayed resorption and divided dosage. Our results indicate that the action of cysteine is not due to a change in resorption but to a chemical inactivation. However, since the inactivation by cysteine is brought about only by prolonged action and in great excess of cysteine, the inactivation may be due to reaction products of impurities.

<sup>4</sup> A supposed difference recently reported by Fevold (8) has been shown by Bischoff (10) to be a question of delayed resorption.

changed the response to the fractionated preparations but the reversal of the response to a minimum effective dose has not yet been attained. This in the final analysis constitutes the only reason for a multiple hormone concept. Since no differences in chemical behavior are forthcoming, and since the specific effects (lack of follicle stimulation and production of antagonism) produced by intraperitoneal injections may be abolished by divided dosage, we must conclude that the criteria existing for differentiating multiple pituitary gonadotropic fractions are so artificial that it were better to abandon them.

## SUMMARY

1. In disagreement with the findings of others, we have found that follicle stimulation can be promoted by intraperitoneal injections of pituitary gonadotropic extracts; the "antagonist" effect can be produced by subcutaneous injection. Either effect is produced by regulation of the rate of resorption.

2. No difference in response to a wide range of chemical reactions was found between the "antagonist" and the gonadotropic factor. The reactions included acetylation, methylation, reaction with  $\beta$ -naphthoquinone sulfonate, with iodine, with diazobenzene sulfonate, or with cysteine, and heat treatment.

3. The existence of the luteinizing and "antagonist" fractions as separate and distinct hormones is not borne out by these observations.

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10. Bischoff, F., *J. Biol. Chem.*, **132**, 35 (1940).



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## LETTERS TO THE EDITORS

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### THE OXIDATION-REDUCTION POTENTIAL OF COENZYME I

Sirs:

The oxidation-reduction potential of cozymase (diphosphopyridine nucleotide) was calculated from the free energies of formation of aqueous *D*-alanine and *D*-glutamic acid based on thermal data, and the equilibria measured by Wurmser and Filitti-Wurmser<sup>1</sup> for pyruvate + 2H<sup>+</sup> + 2(e)  $\rightleftharpoons$  alanine + H<sub>2</sub>O, by Cohen<sup>2</sup> for  $\alpha$ -ketoglutarate + alanine  $\rightleftharpoons$  *D*-glutamate and pyruvate, and by von Euler *et al.*<sup>3</sup> for the reaction  $\alpha$ -ketoglutarate + NH<sub>4</sub><sup>+</sup> + reduced cozymase  $\rightleftharpoons$  glutamate + oxidized cozymase. The value for the potential so calculated is at 30°  $E'_0 = -0.072 - 0.03 \text{ pH} \pm 0.0008 \text{ volt}$ .

Its temperature coefficient is  $\Delta\tilde{E}/\Delta T = -0.00043$ , calculated from the data given by von Euler, Adler, Günther, and Hellström<sup>4</sup> and by Barron and Hastings.<sup>5</sup>

As a check on the accuracy of this calculated value of  $\tilde{E}$  it was used to calculate  $-\Delta\tilde{F}$  for the reaction acetaldehyde (aqueous) + 2H<sup>+</sup> + 2(e)  $\rightarrow$  ethyl alcohol (aqueous) from the equilibrium constant for acetaldehyde + reduced cozymase  $\rightleftharpoons$  ethyl alcohol + oxidized cozymase determined by Negelein and Wulff.<sup>6</sup> This constant (corrected for temperature) and the above value of  $\tilde{E}$  give  $-\Delta\tilde{F}$  for acetaldehyde  $\rightarrow$  alcohol at 25° as 11,620 calories.  $-\Delta\tilde{F}$  calculated from the best thermal and ancillary data is 11,730 calories.

<sup>1</sup> Wurmser, R., and Filitti-Wurmser, S., *Compt. rend. Soc. biol.*, **128**, 133 (1938).

<sup>2</sup> Cohen, P., *Biochem. J.*, **33**, 1478 (1939).

<sup>3</sup> von Euler, H., Adler, E., Günther, G., and Das, N. B., *Z. physiol. Chem.*, **254**, 61 (1938).

<sup>4</sup> von Euler, H., Adler, E., Günther, G., and Hellström, H., *Z. physiol. Chem.*, **245**, 217 (1937).

<sup>5</sup> Barron, E. S. G., and Hastings, A. B., *J. Biol. Chem.*, **107**, 567 (1934).

<sup>6</sup> Negelein, E., and Wulff, H.-J., *Biochem. Z.*, **293**, 351 (1937).



The data of Green and Dewan<sup>7</sup> on the reaction acetoacetate + reduced cozymase  $\rightarrow$   $\beta$ -hydroxybutyric acid + oxidized cozymase, and the  $E'_0$  values of Hoff-Jørgensen<sup>8</sup> for the acetoacetate  $\rightarrow$   $\beta$ -hydroxybutyrate give  $E'_0$  for the cozymase (corrected to 30°) at pH 7.0  $-0.289$ ,  $-0.275$ , and  $-0.274$ . The calculated value is  $-0.282$ .

Ball and Ramsdell<sup>9</sup> recently reported for cozymase a tentative value of  $E'_0$  at pH 7.2 and 25° of  $-0.26$  volt. The difference between this and the above calculated value (more than 0.020 volt) may possibly reside in Ball and Ramsdell's having used a flavo-protein as enzyme. With this protein the ratio of the association constants for the reduced and oxidized cozymase may be very different from those with the proteins of the glutamic acid, ethyl alcohol, and  $\beta$ -hydroxybutyric acid dehydrogenases.

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<sup>7</sup> Green, D. E., and Dewan, J. C., *Biochem. J.*, **31**, 1069 (1937).

<sup>8</sup> Hoff-Jørgensen, E., *Skand. Arch. Physiol.*, **80**, 176 (1938).

<sup>9</sup> Ball, E. G., and Ramsdell, P. A., *J. Biol. Chem.*, **131**, 767 (1939).

## EFFECT OF YEAST EXTRACT AND OTHER SUPPLEMENTS ON THE GROWTH OF CHICKS FED SIMPLIFIED DIETS

Sirs:

Vitamin B<sub>6</sub> deficiency in chicks was produced<sup>1</sup> by use of a simplified diet containing a phosphotungstic acid filtrate of yeast extract as a source of pantothenic acid. It was subsequently observed that such a diet, even when supplemented with vitamin B<sub>6</sub>, was deficient for chicks, depending on the degree of purification of the pantothenic acid preparation. Such a diet was used in this investigation. A slight but definite stimulation of growth was produced by choline in two experiments. Marked stimulation of growth was produced by a crude solution prepared by extracting yeast with 50 per cent ethanol, but a separate experiment showed that the growth-promoting factor was absent from the yeast phosphotungstic acid filtrate. Nicotinic acid produced no additional growth. Basal Diet I consisted of glucose (cerelose) 55 gm., sardine meal (fat-extracted) 20, lard 5, gelatin 5, washed casein 5, lactose 5, fortified sardine oil (3000-A, 400-D) 0.3, ground limestone 1, iodized salt 0.5, bone ash 0.3, KH<sub>2</sub>PO<sub>4</sub> 0.2, MgSO<sub>4</sub> 0.15, ferric citrate 0.1, MnSO<sub>4</sub> 0.1, ZnO 3 mg., CuCO<sub>3</sub> 3 mg., thiamine<sup>2</sup> 0.4 mg., riboflavin<sup>2</sup> 0.6 mg., vitamin B<sub>6</sub><sup>2</sup> 0.3 mg., alfalfa hexane extract equivalent to 1 gm. of alfalfa meal, and yeast phosphotungstic acid filtrate containing 2 mg. of pantothenic acid as measured by biological assay with chicks.<sup>3</sup> Vitamins A and E were fed separately. Eight chicks were placed on each diet at hatching.

In a simultaneous experiment, chicks from the same hatch as in the preceding experiment were fed Diet II consisting of ground polished rice 67.5 gm., washed sardine meal<sup>4</sup> 24, salt mixture<sup>1</sup>

<sup>1</sup> Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **42**, 180 (1939).

<sup>2</sup> Kindly supplied by Merck and Co., Inc., Rahway, New Jersey.

<sup>3</sup> Jukes, T. H., *J. Biol. Chem.*, **117**, 11 (1937); **129**, 225 (1939).

<sup>4</sup> Stokstad, E. L. R., Manning, P. D. V., and Rogers, R. E., *J. Biol. Chem.*, **132**, 463 (1940).

2.5, soy bean oil 3, fortified sardine oil 0.5, thiamine, riboflavin, vitamin B<sub>6</sub>, pantothenic acid preparation, vitamins A and E, and alfalfa hexane extract as above, nicotinic acid 1 mg. This diet is similar to the basal diet of Stokstad, Manning, and Rogers,<sup>4</sup> except for certain additions, notably vitamin B<sub>6</sub>, and for the difference in the respective sources of pantothenic acid.

The tabulated results confirm the observation of Stokstad, Manning, and Rogers<sup>4</sup> that there is a growth-promoting factor in

Group No.	Diet No.	Supplement per 100 gm. diet	Weight, gm.		
			Hatching	15 days	21 days
1	I	1 mg. nicotinic acid	39	68	74
2	I	0.25 gm. choline chloride	38	78	90
3	I	0.25 " " " + 1 mg. nicotinic acid	39	77	89
4	I	4 cc. Yeast Extract 1	39	94	123
5	II	None	41	73	88
6	II	0.25 gm. choline chloride	41	78	94
7	II	4 cc. Yeast Extract 1, treated with nitrous acid	42	80	92
8	II	4 gm. dried brewers' yeast, autoclaved 4 hrs. at 120°	41	83	118
9	II	4 gm. dried brewers' yeast	41	107	152

yeast distinct from the five known members of the vitamin B complex. The results also indicate that the factor is distinct from choline, although choline may exert a slight effect on growth, and that the factor is destroyed by nitrous acid and is somewhat labile to autoclaving. A separate experiment showed that the pantothenic acid content of Yeast Extract 1 was not destroyed by the treatment with nitrous acid.

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## THE BIOLOGICAL FORMATION OF CREATINE

*Sirs:*

In the course of experiments in which nitrogenous compounds containing  $N^{15}$  were given to normal rats creatine was isolated from the muscles. Those samples of creatine obtained from rats given moderate amounts of *dl*-tyrosine, *l*(-)-leucine, ammonia, or urea had only a very small isotope content. When large amounts of ammonia were added to the protein-low diet of immature rats, the creatine had a considerable content of  $N^{15}$ . In contrast to these substances, isotopic glycine, even when given in small amounts, leads to the formation of creatine with a high isotope content. This is in accord with the results obtained by Brand *et al.*<sup>1</sup> in muscular dystrophy. The two preparations obtained after feeding of isotopic glycine and of ammonia respectively are fundamentally different, as shown by degradation. They were heated with strong alkali and the resulting ammonia and sarcosine (isolated as the naphthalenesulfonyl derivative) were subjected to isotope analysis. When isotopic ammonia was given, most of the isotope in the creatine was recovered with the ammonia (derived from the amidine group  $NH_2-C=NH$ )

and only a little was in the sarcosine. The feeding of glycine has the reverse effect: almost all of the isotope was in the sarcosine and practically none in the ammonia. The results indicate that creatine is formed with nitrogen from two different sources: that of the amidine group comes from ammonia (*i.e.* from nitrogen liberated from amino acids) and that of the sarcosine part comes from glycine.

A number of substances suggested to be intermediates in creatine formation have been tested by feeding their isotopic analogues. Sarcosine (prepared by methylation of isotopic

<sup>1</sup> Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., *Am. J. Physiol.*, **90**, 296 (1929). Brand, E., and Harris, M. M., *Science*, **77**, 2007 (1933).

glycine) is as effective a creatine producer as glycine. However, its ingestion leads to the deposition in the proteins of isotopic glycine to the same extent as when glycine is fed. Sarcosine therefore appears not to be a normal intermediate in the formation of creatine but leads to its production by demethylation to glycine. Hydantoic acid (prepared from isotopic glycine) and methylhydantoic acid (prepared from isotopic sarcosine) are completely ineffective; they are not creatine precursors. Guanidoacetic acid (prepared from isotopic glycine with cyanamide) is the most potent producer of isotopic creatine as yet investigated. It yields muscle creatine with about the same isotope content as is found after the ingestion of an equivalent amount of isotopic creatine. These results indicate that the biological formation of creatine involves direct conversion of glycine to guanidoacetic acid, which is then rapidly methylated.

Histidine can be ruled out as a creatine precursor. The imidazole ring of histidine isolated from rats that had produced isotopic creatine contained no isotope. The rôle of arginine as a creatine precursor is being investigated with the aid of isotopic arginine.

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## A SUBSTITUTE FOR BILE SALTS FOR ADMINISTRATION WITH SUBSTANCES POSSESSING VITAMIN K ACTIVITY

*Sirs:*

It is generally accepted that vitamin K and substances possessing vitamin K activity are absorbed from the gastrointestinal tract only in the presence of bile or bile salts. It occurred to us that the value of bile salts in this instance may be due to their non-specific factor of high surface activity. Search for a non-toxic highly surface-active substance, to test this theory, led to the selection of dioctyl sodium sulfosuccinate.

Two batches of capsules were prepared, one batch containing, dissolved in oil, 25 mg. of 2-methyl-1,4-naphthoquinone in each capsule, and the other containing the same amount of the quinone and in addition 60 mg. of dioctyl sodium sulfosuccinate. In each of three cases with prolonged prothrombin times studied to date, the clotting activity in per cent of normal was determined (1) before medication, (2) 48 hours after therapy with 2-methyl-1,4-naphthoquinone (25 mg., three times in 24 hours for 2 days) was begun, and (3) 48 hours after therapy with 2-methyl-1,4-naphthoquinone and dioctyl sodium sulfosuccinate (25 mg. of 2-methyl-1,4-naphthoquinone and 60 mg. of dioctyl sodium sulfosuccinate, three times in 24 hours for 2 days) was begun. In each case the prothrombin time failed to decrease except after treatment with the 2-methyl-1,4-naphthoquinone-dioctyl sodium sulfosuccinate combination.

The observations are indeed few but the uniformity of the results is such as to lead us to record them at this time. Other cases are being studied as they present themselves.

Further consideration arises as to what rôle such surface-active substances may play in fat transport generally.

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## FRACTIONATION OF THE FACTOR PREVENTING NUTRITIONAL ACHROMOTRICHIA

*Sirs:*

In a previous paper by Oleson *et al.*<sup>1</sup> it was shown that certain fractions from liver extract were highly active in preventing achromotrichia in piebald rats on a basal ration low in certain members of the vitamin B complex. In this note we wish to describe further concentration of the factor specifically active in preventing the graying. We have continued to use the ration described previously, although we realize that it is lacking in some of the known growth factors. We have obtained graying in all animals placed on this ration and have not seen any additional symptoms such as the cutaneous lesions noted by György *et al.*<sup>2</sup>

In the fractionation work we used liver extract fractions kindly supplied by Dr. David Klein of The Wilson Laboratories. A 100 gm. lot of the extract was diluted, acidified to approximately pH 3, treated with norit, and the norit eluted with 5 per cent ammonium hydroxide. The eluate was acidified, treated with norit, and the norit eluted with boiling butanol. The butanol was removed *in vacuo*, and the residue acidified to approximately pH 2 and extracted continuously with ether. The ether was removed *in vacuo*, the extract taken up in water, treated with barium hydroxide, and the aqueous solution extracted continuously with chloroform.

The chloroform extract fed at a level equal to 0.5 gm. of liver extract or 70  $\gamma$  of total solids per day prevented graying. This daily dose contained less than 1.5  $\gamma$  of pantothenic acid as assayed by the bacterial method of Snell *et al.*;<sup>3</sup> therefore we believe that

<sup>1</sup> Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **42**, 283 (1939).

<sup>2</sup> György, P., Poling, C. E., and Subbarow, Y., *J. Biol. Chem.*, **132**, 789 (1940).

<sup>3</sup> Snell, E. E., Strong, F. M., and Peterson, W. H., *Biochem. J.*, **31**, 1789 (1937).



the factor preventing nutritional achromotrichia should be clearly differentiated from pantothenic acid. Since factor W appears to be insoluble in chloroform, the above separation differentiates this factor and factor W.

A crystalline material was obtained from the chloroform extract by fractional crystallization with ethyl acetate; when fed at 15  $\gamma$  per rat per day, it prevented the achromotrichia. The residue from the chloroform extract after removal of the crystalline material failed to prevent graying when fed at twice the original level.

The yield of crystalline material obtained was 0.4 mg. per 100 gm. of original liver extract. Further work is now in progress in order to obtain sufficient material for chemical characterization.

In summary, a method for concentrating the factor concerned in the prevention of achromotrichia is described which yields crystalline material. This material is highly active and appears to be distinct from the well recognized members of the vitamin B complex.

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## THE ACTIVATION OF DIPEPTIDASES

*Sirs:*

The instability of dipeptidases from various sources and the difficulties involved in their purification and isolation are well

Substrate	Addition	Hydrolysis,* per cent			
		Dialyzed yeast autolysate	Dialyzed hog intestinal extract	Chick intestinal extract	<i>Bacillus megatherium</i>
Diglycine	None	4	11	40	1
	Mn-cysteine†	34	42	62	48
<i>dl</i> -Prolylglycine	None	6	22	12	0
	Mn-cysteine	42	44	30	36
<i>dl</i> -Leucylglycine	None	18	25	56	1
	Mn-cysteine	50	84	36	50
<i>dl</i> -Alanylglycine	None	58	67	74	1
	Mn-cysteine	32	44	22	90
<i>d</i> -Leucylglycine	None	5	0		0
	Mn-cysteine	58	34		47
Relative‡ activities (rate of diglycine splitting = 1.0)	<i>dl</i> -Prolylglycine	0.6	1.0	0.5	0.8
	<i>dl</i> -Leucylglycine	1.5	4.0	0.9	2.1
	<i>dl</i> -Alanylglycine	3.4	3.2	6.0	3.7
	<i>d</i> -Leucylglycine	0.05	0.06§		1.0

\* Racemic substrates were present in M/15 concentration, diglycine in M/30; pH 7.8 to 8.0. The per cent hydrolysis of one optical component is indicated.

† 0.001 M MnSO<sub>4</sub> plus 0.003 M cysteine.

‡ The relative activities were calculated from hydrolysis values obtained in the presence of activator, except when addition of the activator mixture caused inhibition, in which case the value obtained with enzyme alone was used. The relative activities take into account the varying amounts of enzyme solution and the different incubation periods used in order to obtain hydrolysis values between 20 and 80 per cent. The incubation periods varied from 0.5 to 4 hours, and 0.05 to 0.75 cc. of enzyme solution was used in 3 cc. of reaction mixture.

§ This value was obtained with an undialyzed preparation.

known. In the course of attempts to isolate a yeast dipeptidase, it was found that a combination of metal and reducing agent

was required as an activator. Metal or reducing agent alone may cause a substantial activation, but a combination of the two is usually far superior. For the yeast dipeptidase,  $Mn^{++}$  or  $Fe^{++}$  may serve as the metal, and cysteine, glutathione, or thio-glycolic acid may serve as the reducing agent.  $Mn^{++}$ -cysteine has been the most reliable combination.

Not only yeast dipeptidase, but dipeptidases from hog intestinal mucosa and from many other sources are similarly activated by  $Mn^{++}$  or  $Mn^{++}$ -cysteine. Representative data are given in the accompanying table. It may be seen that the hydrolysis of diglycine and prolylglycine is activated by  $Mn^{++}$ -cysteine in all cases. It has been found that the hydrolysis of all four dipeptides is activatable in some peptidase systems, *e.g.* *Bacillus megatherium*, while the hydrolysis of alanylglycine frequently, and leucylglycine occasionally, is inhibited by  $Mn^{++}$ -cysteine. It is therefore apparent that a very appreciable portion of the activity formerly attributed to the "dipeptidase" and "prolinase" of erepsin and yeast is due to an enzyme characterized by  $Mn$ -cysteine activation.

Contrary to previous literature, many common proteolytic systems are capable of hydrolyzing the "unnatural" peptide *d*-leucylglycine. However, an activator such as  $Mn$ -cysteine is usually required to make this apparent. It has been found that hog erepsin, yeast autolysates, malt extracts, and preparations from *Bacillus megatherium* and *Leuconostoc mesenteroides* are all able to split *d*-leucylglycine at an appreciable rate.

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## GLUCOSE OXIDATION AND PHOSPHORYLATION

*Sirs:*

It was shown<sup>1</sup> that phosphorylation of glucose in kidney extract is linked with the oxidation of succinic to fumaric acid and it was suggested that other substrates whose oxidation involves fumaric acid catalysis may be concerned with phosphorylation. In the experiments with a dialyzed kidney extract presented in the accompanying table addition of a small amount of fumaric acid catalyzes the oxidation of both glucose and pyruvic acid if no fluoride is present. When fluoride is added, fumaric acid no longer catalyzes the oxidation of glucose, while it still does so in the case of pyruvic acid.

1 cc. extract + 0.4 cc. additions	Without fluoride		With fluoride (0.02 M)	
	O <sub>2</sub>	Inorganic P	O <sub>2</sub>	Inorganic P
	<i>c.mm.</i>	<i>mg.</i>	<i>c.mm.</i>	<i>mg.</i>
10 mg. glucose	234	1.60	77	1.55
8 " pyruvic acid + glucose	234	1.60	172	1.08
0.4 " fumaric " + "	959	1.20	266	0.92
0.4 " " + pyruvic acid	804	1.06	646	0.05
+ glucose				

The explanation is that glucose in order to be oxidized by this system must first be transformed to pyruvic acid, a reaction which involves an initial phosphorylation of the glucose molecule, followed by dephosphorylation to pyruvic acid and inorganic phosphate. Fluoride inhibits dephosphorylation and thus prevents the formation of pyruvic acid, the oxidation of which is necessary for the phosphorylation of glucose.

The experiments show that phosphorylation of glucose precedes its oxidation and that fumaric acid catalysis is an essential link

<sup>1</sup> Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).

between phosphorylation and oxidation. The dependence of glucose phosphorylation on the oxidation of pyruvic acid has also been demonstrated in a brain extract.

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## METABOLISM OF *d*-MANNOHEPTULOSE. EXCRETION OF THE SUGAR AFTER EATING AVOCADO

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(Received for publication, January 20, 1940)

Roe and Hudson (1) administered *d*-mannoheptulose<sup>1</sup> to rabbits and concluded from their experiments that this sugar is available to this animal. The same authors (2) later concluded that the rat and the dog were unable to utilize the sugar. Unpublished experiments performed in this laboratory gave no evidence of glycogen formation when mannoheptulose was given by stomach tube to rats. In view of the fact that this sugar occurs free in the avocado, and as this fruit is becoming more popular, it seemed important to study the metabolism of mannoheptulose in man. Consequently, we have observed the excretion of fermentable and non-fermentable sugars after the ingestion of avocado or solutions of mannoheptulose.<sup>2</sup>

### *Methods*

The subjects for the experiments were normal men and women employed in the laboratory. The experiments began at 9.30 a.m., when the fruit or sugar was ingested. Following this, 2 hour specimens of urine were collected up to the end of the 6th hour. An untimed preliminary specimen was included to show that abnormal quantities of sugar were not present. In the first two series of experiments breakfast was allowed and the usual midday meal came within the 2 to 4 hour period. In the last series of

<sup>1</sup> This sugar will be referred to simply as mannoheptulose throughout the paper.

<sup>2</sup> We are indebted to Dr. Carl L. Alsberg for calling our attention to the possibility that sugar might appear in the urine after avocado was eaten.

experiments the subjects had breakfast but did not have the mid-day meal, and the preliminary urine specimen was timed.

Approximate determination of the urine sugar concentration was made by Benedict and Osterberg's picrate method (3) and then appropriate dilutions were made for the determination by the method of Shaffer and Somogyi (4). Interfering substances were removed before this determination by shaking the diluted urine with Lloyd's reagent and permutit. The non-fermentable sugar values were obtained by fermentation with washed yeast (5). In several cases, mannoheptulose was determined by a modification of Roe's fructose method (6). This method<sup>3</sup> was carried out as follows: 2 cc. of urine were diluted 1:10 with 1 per cent acetic acid, treated with 0.2 gm. of acid-washed norit for 5 minutes, and filtered. To 2 cc. of this filtrate were added 4 cc. of a 0.1 per cent alcoholic resorcinol solution and 5 cc. of concentrated HCl solution containing about 1 per cent SnCl<sub>2</sub>. The mixture was heated in a water bath at a temperature of 80° and then read in a colorimeter against a 0.02 or 0.03 per cent mannoheptulose standard treated in the same way. The color produced in the Seliwanoff reaction by mannoheptulose is much less than that given by fructose.

Mannoheptulose was isolated from the Trapp variety of avocado by the methods of La Forge (7) and Montgomery and Hudson (8).

#### EXPERIMENTAL

The Trapp variety of avocado was eaten by ten subjects in quantities ranging from 137 to 214 gm. Avocados are reported to contain from 3 to 7 per cent carbohydrate, but we do not have any values for their mannoheptulose content. The results of the experiments are given in Table I. It is seen that in every case there was a marked increase in the excretion of non-fermentable sugar which generally reached the maximum value in the 2 to 4 hour period. There were at least seven instances in which the concentration of non-fermentable sugar was enough to give a marked reduction with Benedict's qualitative copper reagent. The highest concentration obtained was 0.32 per cent

<sup>3</sup> We are greatly indebted to Dr. Joseph H. Roe for allowing us to use his unpublished method for mannoheptulose determination.

TABLE I  
*Excretion of Urine Sugar after Ingestion of Avocado*

Subject	Avocado eaten	Time	Urine volume	Total fermentable sugar	Non-fermentable sugar		Total mannoheptulose as glucose
					Per 100 cc.	Total	
	<i>gm.</i>	<i>hrs.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
M. E.	138	Before	166	13	20	33	
		0-2 after	136	16	53	72	
		2-4 "	138	80	93	129	
		4-6 "	288	16	44	127	
L. McC.	156	Before	32	1	28	9	
		0-2 after	155	14	64	99	
		2-4 "	67	10	278	186	146
		4-6 "	103	8	121	125	
S. S.	214	Before	460	8	6	28	
		0-2 after	270	21	35	95	
		2-4 "	240	116	76	182	
		4-6 "	326	36	51	166	
M. C. E.	152	Before	117	43	52	61	
		0-2 after	208	26	61	127	
		2-4 "	80	23	211	169	157
		4-6 "	66	8	165	109	85
H. C.	137	Before	48	7	105	50	
		0-2 after	90	9	132	119	77
		2-4 "	82	21	306	251	228
		4-6 "	72	20	238	171	162
L. S.	190	Before	87	11	49	43	
		0-2 after	91	7	141	128	
		2-4 "	76	16	297	226	
		4-6 "	113	14	143	162	
A. L.	136	Before	125	10	38	48	
		0-2 after	112	9	139	156	
		2-4 "	124	15	205	254	225
		4-6 "	131	3	132	173	
J. Q.	167	Before	88	7	45	40	
		0-2 after	44	3	110	48	
		2-4 "	104	23	211	219	196
		4-6 "	142	2	113	160	
J. T.	197	Before	69	12	64	44	
		0-2 after	102	8	148	151	
		2-4 "	148	11	109	161	
		4-6 "	145	10	120	174	
B. C.	153	Before	6	1	49	3	
		0-2 after	77	11	154	119	93
		2-4 "	79	11	318	251	214
		4-6 "	93	8	162	151	115



non-fermentable sugar in the 2 to 4 hour period of subject B. C. Increased excretion of fermentable sugar was observed seven times in the 2 to 4 hour period. The values for mannoheptulose by Roe's method are seen to agree well with the non-fermentable

TABLE II  
*Excretion of Urine Sugar after Ingestion of d-Mannoheptulose*

Subject	Manno- heptu- lose ingested	Time	Urine volume	Total ferment- able sugar	Non-fermentable sugar		Total manno- heptu- lose as glucose
					Per 100 cc.	Total	
	<i>gm.</i>	<i>hrs.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
L. S.	10	Before	61	7	37	23	
		0-2 after	95	25	203	193	175
		2-4 "	61	22	272	166	145
		4-6 "	82	21	221	181	151
		6-7 "	36	3	125	45	
H. C.	5	Before	54	4	69	37	
		0-2 after	85	14	173	147	140
		2-4 "	96	24	195	187	167
		4-6 "	74	20	160	118	99
		6-7 "	36	5	103	37	
J. T.	5	Before	63	10	66	42	
		0-2 after	91	16	202	184	169
		2-4 "	145	22	133	193	
		4-6 "	134	8	88	118	
		6-7 "	60	5	66	40	
J. Q.	10	Before	48	8	42	20	
		0-2 after	64	26	280	179	176
		2-4 "	148	40	152	225	217
		4-6 "	134	20	92	123	
		6-7 "	62	6	61	38	
B. C.	5	0-2 "	73	51	185	135	127
		2-4 "	58	28	330	191	175
		4-6 "	63	23	233	147	131
		6-8 "	102	18	104	107	
		8-10 "	60	7	111	67	
		10-12 "	83	13	80	66	

values when account is taken of the normal content of these substances. 100 mg. of glucose are equivalent to 151 mg. of mannoheptulose when determined by the Shaffer-Somogyi method.

Mannoheptulose in quantities of 5 and 10 gm. was ingested by five of the same subjects who ate avocado. The results are

found in Table II. Again the peak of the excretion of mannoheptulose occurred late, either in the 2 to 4 or the 4 to 6 hour period. In four cases, most of the sugar was excreted by the end of the 6th hour, as indicated by the drop in non-fermentable sugar values to the preliminary levels. In the other case, the fall in non-fermentable sugar content took place in the 8 to 10 hour period. It is interesting that the two subjects who ingested 10 gm. of mannoheptulose excreted no more sugar than did those who took 5 gm. Furthermore, the mannoheptulose excreted after the sugar was taken was comparable in amount to that put out after the avocado was eaten. In this series of experiments, there were at least ten specimens containing enough non-fermentable sugar to give unmistakable reduction of Benedict's sugar reagent. The highest concentration was 0.33 per cent in the 2 to 4 hour specimen of subject B. C. Calculation of the amount of mannoheptulose recovered in the urine shows that about 5 per cent was thus accounted for when 5 gm. were ingested. Small increases in the excretion of fermentable sugar were again obtained.

Another series of experiments on the same subjects as in the second series was undertaken to learn more about the excretion of fermentable sugar after the ingestion of mannoheptulose. In these experiments the subjects went without the midday meal. The results are recorded in Table III. Again it is seen that several of the specimens contained enough non-fermentable sugar to reduce Benedict's qualitative sugar reagent strongly. The highest concentration of non-fermentable sugar occurred in the 6 to 7 hour period of subject B. C. Four of the subjects had the greatest rate of non-fermentable sugar excretion in the 2 to 4 hour period, while the other had practically identical rates in the 2 to 4 and 4 to 6 hour periods. In no case did the rate of non-fermentable sugar excretion of the 6 to 7 hour period approach the preliminary value, showing that the excretion of mannoheptulose was not yet completed. All of the subjects apparently excreted more fermentable sugar after the ingestion of mannoheptulose. However, we believe that these increases are fictitious and are dependent upon the method used in determining fermentable and non-fermentable sugar by yeast. For instance, when approximately 0.2 per cent mannoheptulose was added to a normal urine con-

taining 5 mg. of fermentable sugar per 100 cc., there was an apparent increase of 24 mg. per 100 cc. in this fraction. Similar results were found after the addition of 0.4 per cent mannoheptulose, and 0.2 and 0.4 per cent *l*-xylose to normal urine. It seems probable that the apparent increases in fermentable sugar

TABLE III  
*Excretion of Urine Sugar after Ingestion of 5 Gm. of d-Mannoheptulose*

Subject	Time	Urine volume	Fermentable sugar	Non-fermentable sugar	
		cc.	mg. per hr.	mg. per 100 cc.	mg. per hr.
L. S.	1.5 before	96	4	38	24
	0-2 after	117	7	130	76
	2-4 "	100	16	200	100
	4-6 "	82	11	183	75
	6-7 "	35	6	165	58
H. C.	1.66 before	37	6	76	17
	0-2 after	72	17	202	73
	2-4 "	79	14	233	92
	4-6 "	60	10	215	65
	6-7 "	24	10	190	46
J. T.	1.66 before	82	6	28	14
	0-2 after	111	22	112	62
	2-4 "	110	30	206	113
	4-6 "	86	13	214	92
	6-7 "	33	12	200	66
J. Q.	2.5 before	66	2	43	11
	0-2 after	72	5	135	49
	2-4 "	76	4	220	84
	4-6 "	94	5	124	58
	6-7 "	30	1	120	36
B. C.	2.0 before	43	5	66	14
	0-2 after	100	23	180	90
	2-4 "	87	20	205	89
	4-6 "	62	13	231	72
	6-7 "	26	9	269	70

under such conditions may be due to adsorption on the yeast which is present in great excess.

The fluid intake in the above experiments was purposely restricted in the hope that highly concentrated specimens of urine would be obtained, so that the osazone of mannoheptulose could be isolated and recrystallized. We were successful in obtaining

the characteristic spherulites described by Wright (9) but failed to recrystallize them.

Mannoheptulose has the property of reducing alkaline copper sugar reagents at temperatures under the boiling point. This property serves the useful purpose of helping to identify the sugar. The test of Lasker and Enklewitz (10) for xylulose is thus positive for mannoheptulose, as well as for xylulose and fructose. Mannoheptulose and fructose are positive in the Seliwanoff test but the first is not fermented by yeast while the latter does ferment. Neither mannoheptulose nor xylulose is fermented by yeast and both are positive in Bial's test, while fructose is negative. Mannoheptulose in Bial's test gives a reaction which may be easily mistaken for xylulose, differing in that a fleeting red color is produced before the blue-green color appears. The final reaction product for the two sugars is practically indistinguishable. Application of Tauber's (11) color test for pentoses enables one to decide which sugar is present. Mannoheptulose reduces the common sugar reagents much less strongly than does glucose. We found that 100 mg. of glucose are equivalent to 151 mg. of mannoheptulose when determined by the Shaffer-Somogyi method.

#### DISCUSSION

Our results show that roughly 5 per cent of the mannoheptulose ingested appeared in the urine within 6 hours after 5 gm. had been taken. No greater amount appeared when 10 gm. were ingested. This would indicate that 5 gm. were enough to furnish the quota required by the low coefficient of absorption of the sugar. Excretion of the sugar began to decline after 6 hours. The bulk of the sugar seems to have been disposed of otherwise than through absorption and excretion. It may have been converted to a utilizable carbohydrate, as suggested by the experiments of Roe and Hudson on rabbits, or it may have suffered change in the gastrointestinal tract. Unpublished experiments on rats performed in this laboratory did not show that the sugar was stored as such in the tissues, so we do not consider it likely that such storage occurred in the experiments on man. The results fail to show whether or not mannoheptulose is available to man.

The experimental findings reveal that mannoheptulose may appear in the urine after the avocado is eaten. Furthermore, the concentration of the sugar in the urine may be sufficiently high to

cause reduction of the alkaline copper sugar reagents and lead to a false diagnosis of diabetes mellitus. The simple test of adding 1 cc. of urine to 5 cc. of Benedict's qualitative sugar reagent and allowing the mixture to stand overnight at room temperature will detect xylulose, fructose, and mannoheptulose. Glucose will reduce under these conditions only when its concentration is 2 per cent or higher. This test should be made whenever sugar is found in the urine of a patient seen for the first time. It should perhaps be recalled that xylulose will always be found in the urine of persons having essential pentosuria, but that fructose, in cases of essential fructosuria, and mannoheptulose will occur only when the appropriate foods have been eaten.

#### SUMMARY

It was shown that mannoheptulose appears in the urine of normal persons after the avocado is eaten. The concentration of sugar was high enough to cause reduction of the ordinary sugar reagents, thus becoming another source of confusion in the diagnosis of diabetes mellitus. The peak of excretion of mannoheptulose usually occurred in the 2 to 4 hour period after the avocado was eaten or after the ingestion of the sugar. The rate of sugar excretion began to decrease 6 hours after consumption of the sugar. Approximately 5 per cent of the sugar appeared in the urine during a period of 6 hours after 5 gm. of the sugar were ingested. The fate of the remainder of the sugar was not determined.

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## RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

### XI. THE INFLUENCE OF METHIONINE, CYSTINE, AND CYSTEINE UPON THE PHOSPHOLIPID TURNOVER IN THE LIVER\*

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The rôle of protein in the dietary production and cure of fatty livers has received considerable attention in recent years (1, 2). Casein, like choline, depresses the deposition of excessive amounts of fat in the liver. Not all proteins have identical action in this respect, and this finding has been taken as evidence that some component of the protein molecule is the active lipotropic agent. Thus, Best *et al.* (3) found gelatin to be inactive, whereas Beeston *et al.* (4) showed that edestin possessed some lipotropic activity. Recently several choline-free preparations were assayed in Channon's laboratory (5) and their lipotropic activity was recorded as follows in decreasing order: whale muscle protein, caseinogen, albumin, beef muscle protein and edestin, fibrin and gliadin, gelatin and zein. The first attempt to identify the active component of protein was made by Beeston and Channon (6), who fed rats high fat-low protein diets along with supplements of cystine. No lipotropic action was observed; indeed these workers noted that this amino acid elevated rather than decreased the lipid content of the liver. Tucker and Eckstein (7) then reported that methionine possessed lipotropic properties, and this finding was confirmed by Channon *et al.* (8). Later, Eckstein *et al.* showed that lysine (9) and cysteine and homocystine (10) had no lipotropic effect on the liver.

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Although several views regarding the mechanism of the action of choline have been expressed, the first evidence clearly to establish an association between the action of choline and phospholipid turnover was obtained in this laboratory (11). By the use of radioactive phosphorus as a means of labeling the phospholipid molecule, it was shown that choline speeds up phospholipid turnover in the liver. Choline, moreover, was not the only substance that altered the rate of phospholipid turnover there. Betaine, which resembles choline in its lipotropic effect, was found to stimulate phospholipid turnover (12), whereas cholesterol, the feeding of which results in an increased infiltration of fat in the liver, was shown to depress the turnover of radioactive phospholipid (13). These findings provide evidence that phospholipid turnover is an important intermediary link in the removal or deposition of fat in the liver.

In the present investigation, the same method, namely identification of phospholipid by means of radioactive phosphorus, has been employed to determine the effects of amino acids on the rate of phospholipid turnover. Three amino acids were studied, cystine, cysteine, and methionine.

#### EXPERIMENTAL

The results recorded here were obtained from separate analyses of 184 rats, the data of which are shown in Tables I and II and in Fig. 1. The same procedure was followed throughout in the determination of the effects of each amino acid upon phospholipid activity.

Animals maintained on a standard laboratory diet were fasted for 24 hours and then placed on a high fat diet (11), which consisted of 40 per cent unsalted butter, 50 per cent glucose, 5 per cent extracted casein, and 5 per cent of a salt mixture. Vitamin supplements were also added to the diet before it was fed. The vitamin B complex was furnished as a concentrate obtained from rice bran. A standardized cod liver oil supplied vitamins A and D. After the use of this diet for 3 days, the amino acids were administered. Once the rats were placed on this diet, access to food was permitted during the entire period of observation.

Radioactive phosphorus was injected subcutaneously. Each rat received 1 cc. of an isotonic solution of  $\text{Na}_2\text{HPO}_4$ , which con-

tains 3 mg. of phosphorus. The radioactivity of the sample of phosphorus injected in each rat measured between 0.5 and 1 microcurie. The amino acids were fed by stomach tube at the same time that the phosphorus was injected. Unless otherwise stated, the livers were analyzed for labeled phospholipid at a single interval thereafter; namely, 7.5 to 8 hours after the radioactive phosphorus was injected. At this time the maximum amount of labeled phospholipid was deposited in the liver (11). This interval also provides ample time for the absorption of the amino acids (14, 15).

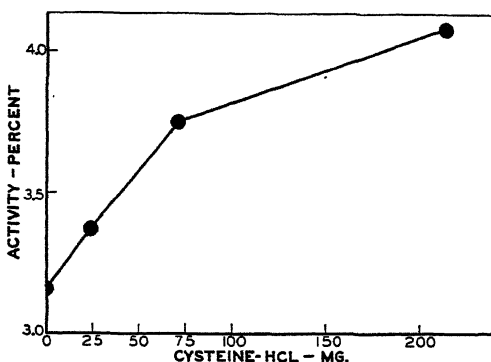


FIG. 1. The effect of varying doses of cysteine hydrochloride upon the level of newly formed phospholipid in the liver. The ordinates refer to per cent of administered labeled phosphorus found as phospholipid of the *whole* liver. Each point is the average of twelve separate analyses. The critical ratios (*d/s.e.d* values) between the group of rats receiving no cysteine and those that were fed 24, 72, and 214 mg. of cysteine hydrochloride were respectively 1.4, 4.2, and 6.3.

The amino acids were fed as 10 or 20 per cent suspensions in 0.5 per cent solution of gum tragacanth. Each animal received by stomach tube 1 cc. of this suspension, which contained 100 or 200 mg. of an amino acid. Control animals received 1 cc. of gum tragacanth solution by stomach tube. In each experiment animals were carefully matched, so that treated animals were compared with control animals of the same weight and sex.

The isolation of the phospholipids from the liver as well as the method by which they were mounted for determination of their radioactivity has been recorded elsewhere (16). Soy bean



lecithin was used as phospholipid carrier and was added to the petroleum ether extract just before precipitation with magnesium chloride and acetone. The amount of lecithin added was such as to give a final mass of 100 mg. in every sample. This was found to be a convenient quantity for mounting on the blotter and was maintained constant in order to rule out possible variations in self-absorption of the radioactivity.

For determination of radioactivity, a blotter (always of uniform size) containing the precipitated phospholipid was placed completely around a small thin walled (0.1 mm.) aluminum Geiger counter, so as to attain a maximum solid angle. 1 microcurie of  $P^{32}$  when measured in this manner yielded  $4 \times 10^5$  counts per minute. Sensitivity of the counter was checked before and after each measurement by means of a standard thorium source.

### Results

*Methionine*—The effects produced on phospholipid turnover by a single feeding of methionine were determined in four separate experiments, which are recorded in Tables I and II. In all, thirty-two treated animals are compared with an equal number of controls. In Table I each rat received 100 mg. of methionine at the end of a 3 day period of fat feeding. In the three experiments shown in Table II each rat received 200 mg. of methionine by stomach tube. This amino acid produced an elevation in the rate of phospholipid turnover. The average activity<sup>1</sup> found 8 hours after the administration of 100 mg. of methionine (Table I) was  $3.41 \pm 0.14$  for the whole liver, as compared with control averages of  $2.70 \pm 0.12$ . In the three experiments recorded in Table II, methionine increased the rate of incorporation of phosphorus into phospholipid by 33, 32, and 41 per cent.

*Cystine*—The effects of this amino acid were tested in four separate experiments involving thirty-two treated rats and thirty-two control animals. Each of the treated animals received a single administration of 200 mg. of *l*-cystine, the results of which are shown in Table II. Increases of 23, 21, 33, and 22 per cent in

<sup>1</sup> Throughout this paper the term phospholipid activity refers to the per cent of administered labeled phosphorus recovered as phospholipid in the whole liver. Control rats and rats that were fed amino acids were carefully paired with respect to weight and sex.

the average phospholipid activities of the whole liver were found after the feeding of this amount of cystine.

*Cysteine*—This amino acid also stimulated the phospholipid turnover in the liver of fat-fed rats. The four separate experiments in which this amino acid was tested involved thirty-two control and thirty-one treated rats (Table II). The average activities, determined at an interval of 8 hours following the ad-

TABLE I  
*Effect of Single Administration of 100 Mg. of Methionine upon Phospholipid Activity of Liver\**

	Control		Methionine†	
	Rat No.	Activity	Rat No.	Activity
	B-1	2.45	M-1	4.00
	B-2	2.45	M-2	3.13
	B-3	3.09	M-3	3.74
	B-4	3.26	M-4	3.23
	B-5	2.87	M-5	3.35
	B-6	2.58	M-6	2.87
	B-7	2.48	M-7	3.81
	B-8	2.44	M-8	3.16
Average activity.....	2.70 ± 0.12		3.41 ± 0.14	
% increase.....			26	
d/s.e.d‡.....			3.9	

\* The percentage of the administered labeled phosphorus found as phospholipid in the whole liver. In these experiments all livers were excised 7.5 hours after the injection of radioactive phosphorus.

† A pure synthetic *dl* preparation.

‡ *d* is the difference between the two means; s.e.d is the standard error of the difference of the means. Critical ratios of 2 and above are usually considered statistically significant.

ministration of 200 mg. of cysteine as the hydrochloride, showed increases of 39, 17, 40, and 25 per cent above the averages for their respective control groups.

*Effect of Varying Cysteine Dosage upon Phospholipid Turnover*—Forty-eight rats were fed the high fat-low protein diet for 4 days and then treated as follows: all rats were injected with 3 mg. of labeled phosphorus. The amount of cysteine hydrochloride fed differed. Twelve rats received none of this amino acid; the other

TABLE II

*Effects of Single Administrations of 200 Mg. of Cysteine, Cystine, and Methionine upon Phospholipid Activity of Liver\**

Experiment No.	Control		l-Cysteine†		l-Cystine‡		Methionine§	
	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity
1. Males, 180 to 200 gm.	Ac-1	2.13	Ce-1	4.32	Ci-1	2.96	M-9	2.66
	Ac-2	1.77	Ce-2	3.00	Ci-2	2.86	M-10	2.83
	Ac-3	3.24	Ce-3	2.52	Ci-3	3.58	M-11	3.33
	Ac-4	2.02	Ce-4	3.29	Ci-4	2.39	M-12	3.60
	Ac-5	2.76	Ce-5	3.61	Ci-5	3.02	M-13	3.03
	Ac-6	2.59	Ce-6	2.62	Ci-6	3.53	M-14	3.39
	Ac-7	2.49	Ce-7	3.00	Ci-7	2.57	M-15	3.31
	Ac-8	1.93	Ce-8	3.90	Ci-8	2.48		
Average activity..	2.37 ± 0.17		3.28 ± 0.22		2.92 ± 0.16		3.16 ± 0.13	
% increase.....			39		23		33	
d/s.e.d. ....			3.3		2.4		3.8	
2. Males, 260 to 300 gm.	Ac-9	2.71	Ce-9	3.04	Ci-9	3.41	M-17	3.96
	Ac-10	2.50	Ce-10	2.58	Ci-10	2.05	M-18	4.31
	Ac-11	2.83	Ce-11	3.85	Ci-11	3.06	M-19	2.78
	Ac-12	2.61	Ce-12	3.58	Ci-12	3.75	M-20	2.76
	Ac-13	2.57	Ce-13	2.60	Ci-13	3.07	M-21	3.57
	Ac-14	2.37	Ce-14	2.81	Ci-14	2.49	M-22	3.99
	Ac-15	3.11	Ce-15	3.30	Ci-15	4.00	M-23	3.31
	Ac-16	2.44	Ce-16	2.96	Ci-16	3.69	M-24	3.10
Average activity..	2.64 ± 0.08		3.09 ± 0.16		3.19 ± 0.24		3.47 ± 0.21	
% increase.....			17		21		32	
d/s.e.d. ....			2.5		2.2		3.8	
3. Females, 170 to 190 gm.	Ac-17	2.12	Ce-17	3.31	Ci-17	5.13	M-25	3.79
	Ac-18	2.99	Ce-18	3.75	Ci-18	3.88	M-26	2.57
	Ac-19	2.99	Ce-19	3.70	Ci-19	3.00	M-27	3.84
	Ac-20	2.51	Ce-20	3.26	Ci-20	3.54	M-28	2.94
	Ac-21	1.27	Ce-21	3.28	Ci-21	3.72	M-29	3.99
	Ac-22	3.87	Ce-22	4.39	Ci-22	3.11	M-30	3.14
	Ac-23	2.68	Ce-23	3.82	Ci-23	2.16	M-31	4.18
	Ac-24	2.24	Ce-24	3.46	Ci-24	2.66	M-32	4.59
Average activity..	2.58 ± 0.27		3.62 ± 0.14		3.40 ± 0.32		3.63 ± 0.24	
% increase.....			40		33		41	
d/s.e.d. ....			3.5		2.0		2.9	

TABLE II—*Concluded*

Experiment No.	Control		L-Cysteine†		L-Cysteine‡		Methionine§	
	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity	Rat No.	Activity
4. Males, 180 to 200 gm.	Ac-25	2.68	Ce-25	3.62	Ci-25	3.51		
	Ac-26	2.66	Ce-26	3.75	Ci-26	3.25		
	Ac-27	3.20	Ce-27	3.79	Ci-27	4.03		
	Ac-28	3.36	Ce-28	3.99	Ci-28	2.70		
	Ac-29	4.16	Ce-29	3.54	Ci-29	4.74		
	Ac-30	2.78	Ce-30	3.48	Ci-30	3.64		
	Ac-31	2.79	Ce-31	3.75	Ci-31	3.98		
	Ac-32	2.07			Ci-32	3.10		
Average activity..	2.96 ± 0.22		3.70 ± 0.06		3.62 ± 0.22			
% increase.....			25		22			
d/s.e.d.....			3.2		2.1			

\* The percentage of the administered labeled phosphorus found as phospholipid in the whole liver. In these experiments all livers were excised 7.5 to 8.0 hours after the injection of radioactive phosphorus.

† Pfanstiehl c.p. grade; fed as the hydrochloride.

‡ Pfanstiehl c.p. grade.

§ A pure synthetic *dl* preparation.

|| *d* is the difference between the two means; *s.e.d* is the standard error of the difference of the means. Critical ratios of 2 and above are usually considered statistically significant.

three groups of twelve rats each received by stomach tube 24 (0.15 milliequivalent), 72 (0.45 milliequivalent), and 214 mg. (1.35 milliequivalents) of it. The amino acid and phosphorus were administered simultaneously and the livers removed for analysis 6.3 hours later. The results show that the magnitude of the activity varies with the dose of cysteine fed. Very little stimulation to phospholipid activity followed the ingestion of 24 mg. 72 and 214 mg. of cysteine hydrochloride, however, resulted in increases of 19 and 29 per cent respectively.

#### DISCUSSION

The present investigation establishes a relation between the metabolism of certain amino acids and the phospholipid activity of the liver. It has been shown here that three amino acids—methionine, cystine, and cysteine—accelerate the phospholipid

turnover in the liver. Although the difference between the labeled phospholipid content in the livers of the treated and untreated rats did not exceed 40 per cent, this difference nevertheless assumes considerable significance when it is recognized that phospholipid metabolism in the liver is a dynamic process in which newly formed phospholipid is constantly replaced.

The stimulation of phospholipid activity produced by methionine is in line with its lipotropic action; *i.e.*, its ability to prevent the deposition of fat in the liver of rats fed high fat-low protein diets (7, 8). But the finding that cystine and cysteine stimulate phospholipid production in the liver is not in line with their negative lipotropic action (6, 10). It should be noted, however, that the method of administering amino acids adopted in the present study differed from those in which their lipotropic action was studied. In the latter, cystine was *fed daily for a period of 2 to 3 weeks*, whereas all observations in the present study were made on livers obtained several hours after a *single* feeding. While an explanation of the lack of uniformity between the lipotropic action and the effect upon phospholipid turnover of cystine and cysteine cannot be offered at the present time, it is nevertheless of interest to call attention here to the pathological changes encountered after prolonged feeding of cystine (17-19). It cannot be inferred, however, that the failure to obtain a lipotropic effect with prolonged feeding of cystine is to be ascribed to its toxicity, for the experiments in which toxic effects were obtained and those in which its lipotropic action was studied were not strictly comparable.

The radioactive phosphorus used in this investigation was supplied by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

#### SUMMARY

The effects of single administrations of methionine, cystine, and cysteine upon the phospholipid activity of the liver of rats fed a high fat-low protein diet were investigated with radioactive phosphorus as indicator.

These sulfur-containing amino acids stimulated the rate of

phospholipid turnover in the liver. Increases in phospholipid activity were observed 6 to 8 hours after single feedings of these three amino acids.

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# THE DISTRIBUTION OF WATER AND ELECTROLYTES IN SKELETAL MUSCLE OF THE DOLPHIN (*Tursiops TRUNCATUS*)\*

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Previous studies on the distribution of water and salts between blood and muscle in terrestrial mammals (dogs) stimulated an analogous study on sea mammals (dolphin, *Tursiops truncatus*). In the past, investigative studies on dolphins did not include chemical analyses of skeletal muscle, and since it has become possible to estimate the relative amounts of extra- and intracellular phases of skeletal muscle (1), this study of these phases in mammals living in sea water has been made.

The principal purposes of this investigation were (1) to determine the distribution of salts and water between serum and muscle, (2) to utilize these data for the quantitative estimation of the relative amounts of the extra- and intracellular phases of skeletal muscle, and (3) to compare these data with those obtained on dogs.

## EXPERIMENTAL

The dolphins used in these experiments had been captured and kept in large circular tanks. Just before they were used, they were transferred to the flume, a smaller tank. Dolphins 2 and 4 had been without food in the flume before being sacrificed. Usually it required from 3 to 5 days in the large tanks for the ani-

\* Part of the expense of this project was defrayed from a grant-in-aid of the Committee on Research in Endocrinology of the National Research Council and from the Biological Grant from the Rockefeller Foundation to the University of Chicago.



mals to overcome the fear resulting from capture. Dolphin 5, a lactating female, ate 10 pounds of mullet the day before it was sacrificed. Dolphin 6 had been in captivity in the large tank for a year. For that reason it was handled more easily than Dolphin 5, only 10 minutes elapsing between the time it was taken from the flume and the taking of the blood sample. These experiments, therefore, include both the animals that were not eating and those that were eating, which is of importance because authorities on this species state that dolphins obtain their fluids almost entirely from their food rather than by drinking water.<sup>1</sup>

Blood was taken from the living animals by heart puncture. The dolphin was then killed by air embolism into the heart, and the rectus abdominis and the sacrospinalis muscles promptly removed. The following determinations were made on the serum: water, chloride, sodium, potassium, calcium, and magnesium. A complete blood and serum analysis was given in a previous paper (2). On the muscle, water, chloride, sodium, potassium, calcium, magnesium, and neutral fat determinations were made.

The blood samples were treated as previously reported (2). After the muscles had been wiped with sterile gauze to remove adherent blood, they were wrapped in waxed paper, chilled, and placed in large thermos jars, which were capped and sent by air express to our laboratories in Chicago. The following morning when the jars were received, the semifrozen muscle was placed on a tile and trimmed to remove free fat and connective tissue. Cross-sections of the muscle were taken from the inner, unexposed part, placed in large glass-stoppered weighing bottles, and minced finely with scissors. The technique for the analyses was the same as that described for kidney tissue (3). The chemical methods also were the same (3, 4). Since the chemical methods already have been given in detail, it is not necessary to repeat them. All

<sup>1</sup> The collection of this material was made possible through the cooperation of the scientific and technical staffs of the Marine Studios, Marineland, St. Augustine, Florida. The location of the Marine Studios is ideal for the capture of dolphins; the trained personnel handles these animals without injuring or unduly exciting them. The efficient service of the Eastern Air Lines and the Air Express of the Railway Express Agency was an indispensable link in the transportation of the samples from St. Augustine or Jacksonville to the laboratories in Chicago.

muscle analyses were corrected for neutral fat. They could not be corrected for circulating blood by a comparison of the amount of hemoglobin in the tissue with that in the whole blood of the same animal because muscle hemoglobin found in the dolphin was too high for this method to be accurate. As a result, all data on the muscle will be expressed on a fat-free basis only.

TABLE I  
*Analyses of Serum and Muscle of Dolphins*

The muscle concentrations are expressed in units per kilo of fat-free muscle.

Dolphin No.		H <sub>2</sub> O	Cl	Na	K	Ca	Mg	Fat	(F)
		<i>gm. per kg.</i>	<i>m.eq. per kg.</i>	<i>m.eq. per kg.</i>	<i>m.eq. per kg.</i>	<i>m.eq. per kg.</i>	<i>m.eq. per kg.</i>	<i>gm. per kg.</i>	
3.* Young female	Serum	887.4	102.9	146.0	3.44	3.92	2.08		
	Sacrospinalis	744.0	10.7	16.7	107.1	2.68	22.8	3.5	89
4. Lactating	Serum	874.3	110.5	156.9	5.29	4.24	2.78		
	Sacrospinalis	731.5	9.5	16.1	99.9	2.40	25.76	0.45	76
5. Near end of lactation	Serum	906.0	105.0	150.5	4.89	4.70	2.06		
	Rectus abdominis	751.5	11.69	20.8	95.2	1.76	17.90	0.90	97
	Sacrospinalis	746.0	10.55	20.1	91.6	1.62	18.34	0.85	87
6. Old female	Serum	906.0	109.5	151.0	4.59	4.58	2.00		
	Rectus abdominis	744.5	10.36	16.3	92.0	1.70	18.90	0.51	83
	Sacrospinalis	741.5	10.01	19.6	98.2	1.62	21.64	0.65	80
Dogs (cf. (1))	Serum	922	109.0	142.0	3.72	2.47			
	σ†	6.0	1.7	4.1	0.40	0.07			
Dogs (cf. (1))	Rectus abdominis	765	21.5	32.4					
	σ†	6.4	2.8	4.8					

\* Weight 80 kilos.

† Standard deviation.

### Results

Table I presents the results of the analyses of serum and skeletal muscle, including the rectus abdominis and the sacrospinalis, from four female dolphins. It will be noted that: (a) The neutral fat in all dolphin skeletal muscle is surprisingly low. Therefore, it is not so essential to express the concentrations in terms of fat-free muscle as in dog muscle. The finding indicates that the excess fat of these animals must be stored almost exclusively in the blubber. (b) The water content and the concentrations of

bases and chloride of the rectus abdominis muscle were not different from the concentrations of the same constituents of the sacrospinalis muscle. (c) The chloride content of the muscle was low. However, if correction could have been applied for the chloride of the blood contained in the tissue, it would have been lower. This may be regarded as additional support for the assumption that chloride in muscle is confined to the extracellular phase. (d) The sodium content of the muscle was higher than the chloride content. Chloride is considered to be only in the extracellular phase of the muscle, and to exist there in the same concentration as in an ultrafiltrate. After allowance is made for sodium in the extracellular phase, based on the chloride calculations, there is "excess sodium" remaining, in varying amounts from 2.5 to 7.0 mM with an average of 4.6 mM. These results are similar to those found in the skeletal muscle of dogs.

*Extracellular and Intracellular Phases of Normal Dolphin Muscle*—From the data of Table I and the fact that the chloride of skeletal muscle is confined to the extracellular phase, the relative proportions of extra- and intracellular phases of skeletal muscle of the dolphin were calculated by means of the equations presented in a preceding paper (1). Briefly, the amounts of extracellular phase ( $F$ ) in gm. per kilo of muscle were calculated from the equation

$$(F) = \frac{(Cl)_m \times (H_2O)_s \times 1000}{1.04 \times (Cl)_s}$$

in which the subscripts  $m$  and  $s$  represent muscle and serum, respectively. From the values for ( $F$ ), the intracellular phase ( $C$ ) per kilo of muscle was estimated by the equation  $(C) = 1000 - (F)$ .

Such a calculation led to the following results. The mass of the extracellular phase ( $F$ ) = 85 gm.,  $\sigma \pm 8$ ; ( $C$ ) = 915 gm.,  $\sigma \pm 8$ .

The data were also used to calculate the percentage of water in the intracellular phase  $\{H_2O\}_c$  by the equation

$$\{H_2O\}_c = \frac{(H_2O)_c}{C}$$

in which  $(H_2O)_c$  represents gm. of intracellular water per kilo of muscle. This calculation led to the value for  $\{H_2O\}_c$  of 720 gm. per kilo of muscle cells,  $\sigma \pm 5$ .

From the data of Table I and the equations, the extra- and intracellular phases of dolphin skeletal muscle were calculated. The results of these calculations are graphically presented in Fig. 1. In all skeletal muscles the extracellular phase was small (varying from a minimum of 75 gm. to a maximum of 97 gm. per kilo of fat-free muscle). These results show that in mammals living in sea water, the extracellular phase amounts to an average of 8.5 per cent of the muscle, and the intracellular phase 91.5 per cent.

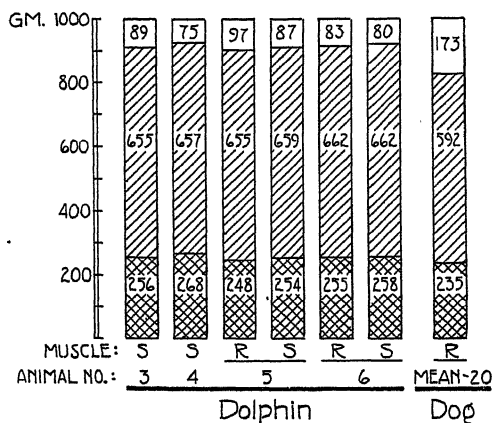


FIG. 1. Graphic representation of the extra- and intracellular phases of skeletal muscle of four dolphins as compared to that of the mean of twenty dogs. The concentrations are presented per kilo of muscle, in which the extracellular phase ( $F$ ) is represented by the unshaded areas, the water content of the intracellular phase ( $H_2O$ )<sub>c</sub> by the simple shaded areas, and the solid content of the intracellular phase ( $S$ )<sub>c</sub> by the cross-hatched areas.  $S$  and  $R$  represent the sacrospinalis and rectus abdominis muscles, respectively.

The phase volumes of skeletal muscle of dolphins were compared with those obtained on the muscle of dogs (Fig. 1), with the following findings.

1. The extracellular phase of 1 kilo of dolphin muscle was about one-half of the value found for dog muscle, proving that sea mammals do not store water in this phase of the skeletal muscle to the extent that the land mammals do.

2. The percentage of water in the intracellular phase in the dolphin skeletal muscle, 72.0,  $\sigma \pm 0.5$ , was the same as in dog

skeletal muscle,  $71.7$ ,  $\sigma \pm 0.5$ . This agreement suggests that the internal composition of the muscle cells of the dolphin is like that of the dog.

#### SUMMARY

1. Normal values for water, chloride, sodium, potassium, calcium, and magnesium in dolphin (*Tursiops truncatus*) serum and skeletal muscle have been determined.

2. The neutral fat in all dolphin skeletal muscle is surprisingly low. Therefore, the excess fat of these animals must be stored almost exclusively in the blubber.

3. From the analytical data obtained on dolphins and the assumption that all muscle chloride is in the extracellular phase, the relative proportions of extra- and intracellular phases of fat-free skeletal muscle have been calculated. The extracellular phase amounts to an average of  $8.5$  per cent,  $\sigma \pm 0.8$ , of the muscle, and the intracellular phase amounts to  $91.5$  per cent,  $\sigma \pm 0.8$ . These values do not agree with those found in dog muscle.

4. The percentage of water in the intracellular phase of dolphin skeletal muscle was found to be  $72.0$  per cent,  $\sigma \pm 0.5$ , which agrees with the value established for the muscle cells of dogs.

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## A RAPID EXTRACTOR FOR URINARY STEROIDS

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This work was undertaken in connection with an investigation being conducted by one of us (J. K. W.) with L. F. Fieser and H. B. Friedgood of the androgens of pathological urines. A continuous extractor has been constructed which meets the requirements of clinical assay work and which appears to have certain advantages over other forms of apparatus designed for the extraction of hormones from urine. This paper includes a description of the extractor and a report of orienting experiments conducted to test its efficiency and rapidity of action as applied to a few urines of low and of high androgen content. Its suitability for general assay work is being investigated further by Dr. Friedgood.

To increase the efficiency of extraction with a solvent lighter than water over that attained in an extractor of the type described by Smith and Smith (1), Gallagher, Koch, and Dorfman (2) provided for the dispersion of the organic solvent (benzene) by causing it to pass through a sintered glass disk, while Talbot and Langstroth (3) introduced an ingenious mechanical stirring device to disperse the solvent benzene into small droplets. The present apparatus utilizes the principle of dispersion with a porous disk as applied to a solvent heavier than water, a combination embodied in an early extractor for general laboratory purposes devised by Friedrichs (4), although this is not mentioned by Wehrli (5) in his review of extractors in which solvents heavier than water

\* Research Fellow on grants from the National Cancer Institute and Eli Lilly and Company to Professor L. F. Fieser.

† Research Fellow on a grant from the Milton Fund of Harvard University to Dr. H. B. Friedgood.

are used. The solvent selected was carbon tetrachloride, which has the practical advantage over benzene or ether of being non-inflammable and comparatively non-toxic and which is regarded by Callow, Callow, Emmens, and Stroud (6) as being superior to benzene as a solvent for the extraction of steroids. The heavy solvent is passed through a sintered glass plate and the fine droplets allowed to fall through a long, narrow column of urine.

*Apparatus (Fig. 1)*—The extractor body *J*, which contains the urine and a lower layer of carbon tetrachloride, is connected by means of the standard taper joint *D* to a unit *G* consisting of a spiral condenser *F* and a sealed-on tube carrying a sintered plate *H*. The boiling flask *A* is fitted to the apparatus by means of a spherical joint *B*, which requires no lubricant and is not easily frozen. The joint is kept closed with an arm type ball clamp with which the flask is supported on a ring-stand. The extractor body is fastened to a ring-stand by a large universal clamp placed near the level of *H*. Stop-cock *K* does not require a lubricant other than the solvent used and permits draining the spent urine remaining after extraction without dismantling the apparatus.

Heating of the boiling flask is best done with a Bunsen burner with a direct, soft flame (an electrically heated aluminum block did not give a sufficiently high boiling rate or permit as easy adjustment of the temperature). The vapors ascend through the tube *C* and enter the condenser chamber through a hole drilled in the male section of joint *D*. The condensate then falls through tube *I* and is dispersed by the sintered glass plate *H* which dips under the surface of the urine. The solvents separate in the lower part of the chamber; the carbon tetrachloride containing hormone returns to the boiling flask through stop-cock *K* and tube *L*. Tube *E* equalizes the pressure between the extractor body and the condenser system and also allows accumulated water to overflow and return to the main body of the urine.

The efficient and compact spiral type of condenser adequately takes care of the vapor at the desired maximum boiling rate. In trials with a spiral having only sixteen turns it was possible to maintain a flow of more than 10 liters of carbon tetrachloride per hour with cooling water at either 5° or 30°.

From a trial of dispersion plates of different porosity, it was concluded that a coarse plate is better than a fine one. With a

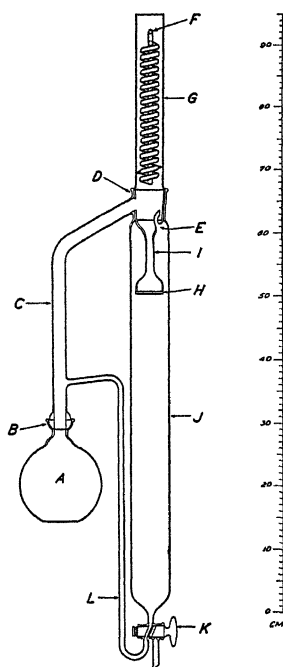


FIG. 1. Assembly of urine extractor. *A*, 1 liter flat bottom boiling flask; *B*, spherical joint 35/20 (Ace Glass, Inc., Vineland, New Jersey; Scientific Glass Apparatus Company, Bloomfield, New Jersey); *C*, vapor tube, 20 to 22 mm. inside diameter; *D*, special 50/50 standard taper joint with an 18 to 20 mm. hole drilled in the side of the male section; *E*, overflow-equalizing tube, 7 mm. outside diameter. The outlet hole is 7 to 9 mm. below the vapor inlet; *F*, coiled tube condenser of tubing of 8 mm. outside diameter with both inlet and outlet at the top and facing to the back. The diameter of the spiral is 36 to 38 mm. and it is centered by three small knobs at the lower end of the coil. The clearance between the coil and side wall is 3 to 4 mm.; *G*, condenser jacket, 42 to 46 mm. inside diameter; *H*, porous glass disk, 41 to 43 mm. outside diameter, with average pore diameter of 85 to 145  $\mu$  (Ace Glass, Inc.); *I*, liquid connector tube, 13 to 15 mm. outside diameter. The length is such that plate *H* is 130 to 140 mm. below the vapor inlet hole; *J*, extractor body, 60 to 62 mm. inside diameter, 64 to 66 mm. outside diameter; *K*, 3-way 4 mm. bore stop-cock with interchangeable No. 17-F plug (Scientific Glass Apparatus Company, No-Lub catalogue No. C-880). This permits the extraction to be carried out without contamination by stop-cock grease. The same result can be obtained with an ordinary stop-cock by a final light grinding with jewelers' rouge suspended in either oil or water; *L*, return tube, 9 mm. outside diameter. See the calibration procedure for the vapor tube connection.



fine plate it is necessary to provide a greater fluid head by lengthening tube *I*, with consequent decrease in the effective volume available for the urine sample, and even so the solvent flow cannot be maintained at the maximum boiling rate. A fine plate also gives rise to more trouble with the emulsions which tend to form in the extraction of urine with a dispersed solvent at a high rate of flow, and this may necessitate an increase in the space provided at the bottom of the extractor for the separation of layers. The water content of such emulsions, to be sure, is low, and in one test with a fine plate the emulsion was allowed to siphon over with no apparent harm. The tendency to form persistent emulsions is greatly reduced by using a coarse plate, and at the resulting high rate of flow the droplets are still very small and appear in a shower extending to all parts of the tube. Another important factor is the temperature, emulsions being more prone to form in the cold than at the temperature of about 60° which is reached after the extractor has been in operation. If the urine is introduced cold, the combination of an unfavorable temperature and a high initial steroid content may cause considerable trouble, but the difficulty is easily met by first warming the urine to 60°. Under these conditions, the emulsion usually breaks a short distance below the interface, and even with dark pathological urines very rich in steroids it is only necessary to moderate the boiling in the early stages of the extraction in order to prevent the emulsion from being carried over.

Prausnitz (7) recommends that a sintered glass plate be wet first with the organic solvent, while Friedrichs (8) states that smaller drops are obtained by wetting it with the aqueous solution. In our experience with the present extractor, the particle size appeared to be the same in either case after a short period of operation.

*Assembly of Apparatus*—The extractor is designed to accommodate a fixed volume of hydrolyzed urine in each operation, for this makes for simplicity of construction. The volume decided upon, in relation to the total capacity of the extractor tube, fixes the level at which the return tube *L* should enter the vapor tube *C*, and since this is the last connection to be made in assembling the apparatus, the proper point of entrance must be determined by experiment. The extractor body *J*, with the vapor tube *C* and

the stop-cock *K* sealed in place but not yet directly connected, is fitted with the condenser unit and charged with 1175 cc. of water at room temperature and enough carbon tetrachloride is added to cause the sintered glass plate *H* to dip 10 to 15 mm. into the water. A rubber tube is connected to one of the outlets of stop-cock *K* and slowly lowered from a vertical position until a point is reached where carbon tetrachloride begins to run out. This is selected as the proper point for the entrance of the return tube *L* into *C*.

*Operation*—In the usual case a 1 liter sample of urine is treated with 150 cc. of concentrated c.p. hydrochloric acid and the mixture boiled for 10 minutes under a reflux. The extractor is charged with 250 cc. of carbon tetrachloride and the hydrolyzed urine is cooled to 50–60° and introduced through the open joint *D*, a funnel being used to prevent spilling of the liquid into *C* or onto the ground joint, for this may cause the joint to stick. The condenser unit is inserted and a rapid stream of cooling water started. Flask *A* is charged with 500 cc. of carbon tetrachloride and heated with the direct flame of a Bunsen burner. With normal urines boiling can be conducted at the maximum rate from the start, but with pathological urines containing suspended solid the heating should be moderate at the outset, with a gradual increase in the course of about 15 minutes, when the initial emulsion coagulates and full heat can be applied.

In the test experiments the extraction was interrupted at suitable periods and the extract in the boiler transferred with rinsing to a distillation flask and replaced by fresh solvent. The solvent was completely removed from the extract by distillation at diminished pressure and the residue was dissolved in 200 cc. of ether and the solution washed in a separatory funnel with five 25 cc. portions of 10 per cent sodium hydroxide solution, the washings being discarded. The ethereal solution was evaporated to dryness at reduced pressure and the residue dissolved in 95 per cent ethanol and transferred to a small volumetric flask of size proportional to the estimated sterone content.

We are indebted to Dr. H. B. Friedgood and Miss R. A. Berman of the Endocrine Laboratory of the Peter Bent Brigham Hospital for making colorimetric assays of the extracts and for carrying out parallel extractions of some of the urines with benzene in the apparatus of Smith and Smith (1). The assays were con-

ducted by a modified Zimmerman procedure (9) similar to that of Callow *et al.* (10) and the results are expressed in mg. equivalents of androsterone.

The results listed in Table I show that with urines of widely varying androgen content the new apparatus extracts somewhat more sterone in  $\frac{1}{2}$  hour than the Smith and Smith benzene extractor does in 22 hours. With the urines in or near the normal range of hormone content, the material removed in the half hour period is about 95 per cent of that extracted in a period twice as

TABLE I  
*Performance of Extractor; Mg. of Sterone Extracted in Successive Periods  
(Calculated As Androsterone)*

Urine sample	Total extraction time								
	30 min.	45 min.	60 min.	75 min.	90 min.	120 min.	150 min.	17 hrs.	22 hrs.
A	16.9		0.9						
B	18.9	0.6	0.2						
B <sub>1</sub> . Benzene extractor									15.7
C	21.0		0.6						
D	21.0	0.8	0.5		0.3				
E	21.4		0.9	0.2					
"	21.7		1.0	0.4					
E <sub>1</sub> . Benzene extractor									18.6
F	23.2		1.0		0.9	0.4		0.8	
G		25.4	0.7	0.2			0.3		
H	235								
H <sub>1</sub> . Benzene extractor									230
J	394		88		55	31	25	40	

long and doubtless represents nearly the entire amount present. This performance is somewhat more rapid than that attained in the extractor of Talbot and Langstroth (3). The apparatus has the added advantage of being rugged in construction and can be conveniently refilled. Since spent urine can be replaced by a fresh charge without dismantling the apparatus, a large quantity of urine can be extracted easily and the total hormone accumulated in the boiling flask.

With the specimen of urine (Sample J) of extremely high androgen content the steroid extracted in  $\frac{1}{2}$  hour is 80 per cent of

that removed in 1 hour and 62 per cent of the total accumulated in 17 hours, which may still be somewhat short of the actual total present. The comparative slowness of extraction may be due both to the high steroid content and to occlusion of hormone in the pigmented solid dispersed in the urine.

Since in our apparatus the rapidly flowing solvent maintains the urine at a steady temperature of about 60°, it may be practicable to combine the hydrolysis step with the extraction and so minimize a possible destructive action of the hot acid on certain of the hormones. This possibility is being investigated.

#### SUMMARY

A rapid and efficient apparatus is described for the extraction of steroids from urine embodying the principle of causing carbon tetrachloride to pass through a porous disk and fall in fine droplets through a long column of urine. Colorimetric androgen assays of normal and abnormal urines demonstrate the satisfactory performance of the apparatus. With urines in the normal range, the bulk of the androgen fraction is extracted in  $\frac{1}{2}$  hour.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### LX. CONCERNING THE FIRMLY BOUND LIPIDS OF THE AVIAN TUBERCLE BACILLUS\*

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It has been known for a long time that acid-fast bacteria contain a certain percentage of lipids that are so firmly combined in the cellular structure that they cannot be removed by extraction with neutral solvents such as alcohol, ether, or chloroform, and this material we have designated by the term firmly bound lipids. The firmly bound lipids can be removed easily from the bacterial residues which remain after exhaustive extraction with neutral solvents by treatment with very dilute hydrochloric acid, followed by extraction with ether and chloroform.

Previous reports from this Laboratory have dealt with the chemical composition of the firmly bound lipids isolated from the human tubercle bacillus (1) and from the so called leprosy bacillus (2). The firmly bound lipids of the human tubercle bacillus could be separated into filtrable and unfiltrable fractions by forcing a chloroform or ether solution of the lipid through a Chamberland filter. On analysis the composition of the two fractions was found

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to differ mainly in the percentage of carbohydrate; the unfiltrable lipid contained about 50 per cent, while the filtrable lipid contained about 25 per cent of carbohydrate. In examining the firmly bound lipids of the leprosy bacillus (2) no unfiltrable lipid was obtained, but the fraction that was analyzed contained about 40 per cent of a specific polysaccharide.

In continuation of the studies mentioned above, we have recently made an examination of the firmly bound lipids of the avian type of tubercle bacilli. The material could be separated into filtrable and unfiltrable fractions by forcing a chloroform solution of the lipids through a Chamberland filter. In the present report we present the result of this investigation, which is divided into the following five parts: (I) Extraction and separation of the firmly bound lipids of the avian tubercle bacillus; (II) Composition of the unfiltrable portion of the firmly bound lipids; (III) Composition of the polysaccharide of the unfiltrable lipid; (IV) Composition of the filtrable portion of the firmly bound lipids; (V) Pyrolysis of the hydroxy acids of the filtrable portion of the firmly bound lipids.

In comparing the cleavage products of the firmly bound lipids (1) with those of the chloroform-soluble wax (3) of the human tubercle bacillus, we found that they were of the same nature and consisted essentially of mycolic acid (4) and a specific polysaccharide. The polysaccharides from both products gave on hydrolysis mixtures of reducing sugars consisting of mannose, *d*-arabinose, and galactose.

In the case of the avian tubercle bacillus we have found that the chloroform-soluble wax and the firmly bound lipids differ entirely in composition. The chloroform-soluble wax, as shown by Reeves and Anderson (5), contained mainly hydroxy acids of high molecular weight, combined with the disaccharide trehalose and about 10 per cent of unsaponifiable matter. The latter consisted principally of *d*-eicosanol-2 but a small amount of *d*-octadecanol-2 was also present. As is shown in the present investigation, the firmly bound lipids contain mainly hydroxy acids of high molecular weight, *d*-eicosanol-2, and a specific polysaccharide which on hydrolysis gives mannose, *d*-arabinose, galactose, and traces of glucosamine and inosite.

## EXPERIMENTAL

*I. Extraction and Separation of the Firmly Bound Lipids of the Avian Tubercle Bacillus*

*Extraction with 25 Per Cent Alcohol*—For the present experiments we used 500 gm. of dried bacterial cells which had been extracted with alcohol, ether, and chloroform by Anderson and Roberts (6). The cells were first treated with 2 liters of 25 per cent alcohol for 6 hours at 40°, after which the cells were filtered off and washed with 25 per cent alcohol. Five further extractions for 2 hours at 40° with 1 liter of 25 per cent alcohol were made. The cells were filtered off after each extraction and washed with 25 per cent alcohol.

The extracts were combined, filtered through a Mandler filter, and concentrated under reduced pressure to a suitable volume. The solution was used for the isolation of a lactoflavin concentrate and for the preparation of a polysaccharide. These products were used in other experiments.

*Treatment with Acidified Alcohol-Ether*—The moist bacterial cells, after the extractions mentioned above, were digested under a reflux for 3 hours at 50° with 2 liters of equal parts of alcohol and ether containing 55 cc. of concentrated hydrochloric acid. After the mixture had cooled, the cells were filtered off and the filtrate was concentrated *in vacuo* to a volume of about 250 cc. The solution was diluted with an equal volume of water and extracted thoroughly with ether. The ethereal extract, after being washed free of acid with water, dried over sodium sulfate, and filtered through a Chamberland filter, was evaporated to dryness. The residue was a brownish oil which weighed 7.58 gm. This fraction has not been further investigated.

*Extraction with Ether-Chloroform*—The bacterial cells were next digested at 40° for 1 hour under a reflux in 1 liter of a solution containing 2 parts of ether and 1 part of chloroform, and this was followed by a second extraction in the same manner. The cells were next digested five times, with 1 liter of ether each time, and warming at 35° for 2 hours under a reflux. The cells were filtered off after each extraction and washed with ether. The last ether extract contained a mere trace of lipids. The extracts were con-



centrated at a temperature of 45° by means of a current of carbon dioxide to a volume of about 1 liter. The solution was shaken with solid sodium bicarbonate in order to remove hydrochloric acid, after which it was filtered through paper and then through a Chamberland filter, under carbon dioxide pressure. The Chamberland filter was washed four times with 100 cc. portions of chloroform.

The filtrate and washings on evaporation to dryness gave 32.88 gm. of a solid residue which will be termed *filtrable lipid*. The substance was dissolved in 300 cc. of ether and 300 cc. of alcohol were added, whereupon a heavy white precipitate separated. The precipitate was filtered off, washed with alcohol, and dried *in*

TABLE I  
*Firmly Bound Lipids Extracted from 500 Gm. of Defatted  
Avian Tubercle Bacilli*

	Weight	Per cent	P	N	M.p.
	gm.		per cent	per cent	°C.
Filtrable lipid in acidified alcohol-ether extract.....	7.58	1.51			Oil
Filtrable lipid in ether-CHCl <sub>3</sub> extracts.....	32.88	6.57	0.13	None	60-90
Unfiltrable lipid in ether-CHCl <sub>3</sub> extracts.....	13.73	2.74	0.70	0.47	190-200
Total bound lipids.....	54.21	10.83			

*vacuo*. The substance was a snow-white powder, which weighed 21.5 gm. and melted indefinitely, sintering at 60° and melting at 90°. On further heating it gradually turned yellowish and decomposed at about 200°. The substance contained 0.13 per cent of phosphorus but it was free from nitrogen. A portion of this fraction was saponified and the cleavage products were examined, as will be described in Part IV.

The filtrate and washings from the above substance were combined and evaporated to dryness under reduced pressure. The solid residue was not further examined.

*Unfiltrable Lipid*—The Chamberland filter, through which the extract mentioned above had been filtered, was found to contain some gum-like material which was dissolved in ether, giving a

cloudy solution. The ether was evaporated and the residue was dissolved in ligroin and this solution was centrifuged at high speed in order to remove suspended bacterial cells. The clear supernatant solution was decanted and evaporated to dryness *in vacuo*. The residue was dissolved in 100 cc. of ether and an equal volume of alcohol was added, which caused a heavy white precipitate. The precipitate was collected, washed with alcohol, and dried *in vacuo*. The product was a white granular powder which weighed 13.41 gm. The filtrate and washings on evaporation to dryness gave a residue which weighed only 0.32 gm. and which was not further examined. The principal portion of the unfiltrable lipid was examined, as will be described in Part II. The several fractions of firmly bound lipids isolated from the avian tubercle bacillus are summarized in Table I.

## *II. Composition of the Unfiltrable Portion of the Firmly Bound Lipids*

The unfiltrable fraction of the firmly bound lipid obtained as mentioned above melted with decomposition between 190–200°. On analysis the substance was found to contain 0.47 per cent of nitrogen and 0.70 per cent of phosphorus. Without any further attempt at purification a portion of the substance was saponified and the cleavage products were determined as described below.

*Saponification of Unfiltrable Lipid*—12.43 gm. of the substance were dissolved in 110 cc. of benzene and 30 cc. of 5 per cent alcoholic potassium hydroxide were added. This gave a perfectly clear solution, but on heating a cloudiness appeared and a precipitate separated gradually. After being refluxed on a water bath for 30 hours, the mixture was allowed to stand at room temperature until the precipitate had settled. The supernatant was decanted and the insoluble material was collected on a Buchner funnel and washed with benzene. The benzene-insoluble substance represented the carbohydrate component and was examined, as will be described in Part III.

*Ether-Soluble Saponification Products*—The benzene-alcohol solution was freed of excess of potassium hydroxide by means of carbon dioxide and the potassium carbonate was filtered off, washed with benzene, and discarded. The filtrate, after being concen-

trated to a volume of about 75 cc., was mixed with 250 cc. of ether and washed, first with dilute hydrochloric acid and afterwards with water, until the washings were neutral to litmus.

The aqueous washings were neutralized with potassium hydroxide and concentrated to dryness. The residue was examined for glycerol in the usual manner, but none was found.

The ethereal solution was dried over sodium sulfate and evaporated to dryness. The residue was a slightly yellowish wax-like solid which weighed 4.3 gm. Unfortunately about one-half of the material had been lost during the isolation process. In a separate experiment it was found that the ether-soluble constituents liberated on saponification amounted to 64 per cent of the unfiltrable lipid.

#### *Ether-Soluble Constituents*

*Isolation of Hydroxy Acids*—The total ether-soluble material, 4.3 gm., was dissolved in 20 cc. of ether and 50 cc. of alcohol were added, which caused a white amorphous precipitate, which was filtered off, washed with alcohol, and dried *in vacuo*. On concentration of the filtrate until the ether was removed, an additional small amount of precipitate separated and was filtered off. The alcohol-insoluble product consisted of hydroxy acids and weighed 3.28 gm., corresponding to 76.2 per cent.

The substances which remained dissolved in the alcoholic filtrate were examined, as will be described later.

The crude hydroxy acid melted at 56–58° and the equivalent weight determined by titration was 946. The substance was very slightly soluble in cold ether but was readily soluble in warm ether. It was practically insoluble in cold alcohol or acetone. The substance after six precipitations from ethereal solution by cooling gave Fraction I, 0.5 gm., m.p. 64–67°,  $[\alpha]_D$  in  $\text{CHCl}_3$  +0.9°, mol. wt. by titration 1156.

From the mother liquors on concentration and precipitation with acetone two fractions were obtained which had the following properties.

*Fraction II*—0.9 gm., m.p. 60–61°,  $[\alpha]_D$  in  $\text{CHCl}_3$  +2.3°, mol. wt. by titration 965. Found, C 81.57, H 13.82.

*Fraction III*—1.1 gm., m.p. 59–60°,  $[\alpha]_D$  in  $\text{CHCl}_3$  +3.9°, mol. wt. by titration 981. Found, C 81.74, H 13.76.

The simplest formula calculated from the analytical values is  $C_{71}H_{142}O_3$  (1042). Calculated, C 81.76, H 13.62. The results obtained would indicate that the fractions were mixtures of higher hydroxy acids that were very similar in composition but which differed in melting point and optical rotation.

*Isolation of Lower Fatty Acids*—The alcoholic filtrate from the hydroxy acids was precipitated by the addition of an alcoholic solution of lead acetate. The lead salt was freed of lead in the usual manner and gave 0.1 gm. of a solid fatty acid which was not further examined.

The filtrate from the lead salt was concentrated *in vacuo* nearly to dryness, transferred to a separatory funnel with ether, and washed first with dilute hydrochloric acid and afterwards with

TABLE II  
*Composition of Ether-Soluble Constituents of Unfiltrable Lipid*

	<i>per cent</i>
Hydroxy fatty acids.....	76.2
Lower fatty acids.....	5.6
Neutral material, <i>d</i> -eicosanol-2.....	15.5
Total recovered.....	97.3

water until the washings were neutral to litmus. The ethereal solution was then extracted with 2 per cent potassium hydroxide. The alkaline extract yielded 0.14 gm. of a liquid fatty acid which was not further examined.

*The Neutral Material. Isolation of d-Eicosanol-2*—The ethereal solution after the above extractions was evaporated to dryness and left a neutral crystalline residue which weighed 0.67 gm. The substance was recrystallized several times from ethyl acetate and was obtained in rosettes of colorless prismatic needles. The crystals melted at 62–63° and in ether solution showed  $[\alpha]_D = +7.1^\circ$ . The crystal form and properties identify the substance as *d*-eicosanol-2 (7).

The results of the examination of the ether-soluble constituents are summarized in Table II.

### *III. Composition of the Polysaccharide of the Unfiltrable Lipid*

The material which had separated from the saponification mixture was dissolved in 50 cc. of water and the solution was made

faintly acid with acetic acid. A solution of neutral lead acetate was added in slight excess and the precipitate which formed was filtered off, washed with water, and discarded. The filtrate after being decolorized with norit was precipitated by the addition of basic lead acetate and ammonia. The lead salt was filtered off, washed, suspended in water, and decomposed with hydrogen sulfide. The lead sulfide was removed and the clear filtrate was concentrated to a thick syrup *in vacuo*. The syrup was dehydrated by grinding under absolute alcohol until a fine, nearly white powder was obtained. The powder, after it had been filtered off, washed with absolute alcohol, and dried, weighed 3.9 gm., which is equal to 31.3 per cent of the lipid.

The substance gave pentose color reactions with orcinol and phloroglucinol and also a reddish color with resorcinol. No color reaction was obtained with naphthoresorcinol and the substance did not reduce Fehling's solution.

On hydrolysis with 5 per cent sulfuric acid the maximum reduction was reached in  $3\frac{1}{2}$  hours and amounted to 50.1 per cent, calculated as glucose.

With immune horse serum the substance gave a precipitin reaction in dilutions up to 1:500,000.

On analysis the following values were obtained: phosphorus 1.04, nitrogen 1.81, and ash 2.04 per cent.

*Total Pentose Determination according to Tollens' Method*—0.5198 gm. of the substance gave 0.1399 gm. of phloroglucide. According to Kröber's table this value corresponds to 0.1598 gm. of arabinose, which is equal to 30.7 per cent of the polysaccharide.

The phloroglucide did not lose in weight on treatment with warm alcohol, thus indicating the absence of methylpentose.

The results of the analyses indicate that the substance is a polysaccharide which gives pentose and other reducing sugars on hydrolysis.

The solution which remained after the furfural had been distilled off was decolorized with norit and evaporated to dryness *in vacuo*. The only substance that could be identified in the residues was inosite, of which 4.5 mg. were obtained. The substance crystallized in the characteristic colorless prismatic needles, gave the Scherer reaction, and melted at 224–225°.

*Hydrolysis of the Polysaccharide*—A portion of the polysac-

charide weighing 2.1 gm. was hydrolyzed by refluxing with 25 cc. of 5 per cent sulfuric acid for  $3\frac{3}{4}$  hours and the cleavage products were examined as follows: The solution was first extracted three times with chloroform but the extract on evaporation to dryness left only a trace of residue. It is evident, therefore, that neither fatty acids nor other chloroform-soluble substances were present. The solution was next decolorized with norit, after which the sulfuric and phosphoric acids were removed quantitatively with barium hydroxide. The colorless filtrate was concentrated *in vacuo* to a volume of 20 cc.

*Test for Mannose*—To 1.0 cc. of the above solution was added some phenylhydrazine dissolved in a few drops of alcohol. A crystalline precipitate appeared almost immediately, indicating the presence of mannose. The test solution was, therefore, combined with the main solution and to the latter was added 1.0 gm. of phenylhydrazine dissolved in 1.0 cc. of alcohol. The mannose phenylhydrazone crystallized very rapidly and after standing for several hours in the refrigerator the crystals were filtered off, washed with water and with alcohol, and dried *in vacuo*. The crystals weighed 0.8623 gm., corresponding to 0.5748 gm. or 27.3 per cent of mannose. After being recrystallized from 80 cc. of 60 per cent alcohol, massive, nearly colorless crystals were obtained, which weighed 0.65 gm. The powdered crystals when rapidly heated melted with decomposition at 196–197°. A mixture of the above substance with authentic mannose phenylhydrazone melted with decomposition at the same temperature.

*Isolation of d-Arabinose*—The filtrate from the mannose phenylhydrazone was freed of excess phenylhydrazine by means of benzaldehyde in the usual manner and the filtrate from the benzaldehyde phenylhydrazone was repeatedly extracted with chloroform, after which the solution was concentrated *in vacuo* to a thick syrup.

Unfortunately, a small amount of the syrup was lost through an accident. Consequently the amount of the other reducing sugars could not be determined quantitatively.

The syrup was stirred up with 10 cc. of 75 per cent alcohol, when a small amount of material did not dissolve but remained as a syrupy mass. After standing for some time the clear supernatant solution was decanted.

The insoluble portion, after being dried *in vacuo*, weighed 0.35 gm. and was examined, as will be described later.

To the decanted solution was added 1.0 gm. of benzylphenylhydrazine and the mixture was warmed gently until a clear solution was formed. The hydrazone began to crystallize within a few minutes. After the mixture had stood in the refrigerator overnight, the crystals were filtered off and washed with cold 75 per cent alcohol and with ether. The product was snow-white and after being dried *in vacuo* weighed 0.9216 gm., corresponding to 0.4189 gm. or 19.9 per cent of *d*-arabinose. The product melted at 174° and gave no depression of the melting point when mixed with pure *d*-arabinose benzylphenylhydrazone. After being recrystallized from 20 cc. of 75 per cent alcohol, snow-white, fine needle-shaped crystals were obtained which weighed 0.83 gm. The crystals melted at 174–175° and did not depress the melting point of an authentic sample of *d*-arabinose benzylphenylhydrazone.

*Galactose Benzylphenylhydrazone*—The mother liquor from the arabinose benzylphenylhydrazone was concentrated under reduced pressure until the alcohol was removed. The precipitate which separated was filtered off, washed with water, and dried *in vacuo*. The product was twice recrystallized from acetone and gave fine, colorless, needle-shaped crystals which weighed 0.1460 gm., corresponding to 0.0730 gm. or 3.5 per cent of galactose. The crystals melted at 157–158° and there was no depression of the melting point of authentic galactose benzylphenylhydrazone which also melted at 157–158°.

*Isolation of Inosite*—The mother liquor from the crude galactose benzylphenylhydrazone was freed of excess of benzylphenylhydrazine by means of benzaldehyde in the usual manner, after which the solution was concentrated to dryness *in vacuo*.

The residue was dissolved in a little water and alcohol was added carefully until the solution was faintly cloudy. After the solution had stood crystals separated, on scratching, which were collected and recrystallized in the same manner. The colorless prismatic crystals weighed 15 mg. The crystals gave the Scherer reaction and melted at 224°. The properties identify the substance as inosite.

*Isolation of an Organic Phosphoric Acid*—The material which

was insoluble in 75 per cent alcohol and which was referred to above weighed 0.35 gm. It gave the Scherer reaction, thus indicating the presence of inosite, and after being decomposed by the Neumann method gave a positive test for phosphorus. An organic phosphoric acid was isolated as follows: The substance was dissolved in a little water and lead acetate was added in excess. The insoluble lead salt which separated was filtered off, washed with water, and decomposed in aqueous suspension with hydrogen sulfide. After removing the lead sulfide, the filtrate was concentrated under reduced pressure to a syrup. The latter was strongly acid in reaction and after drying *in vacuo* weighed 47 mg. The substance was insoluble in alcohol but could not be obtained in crystalline form. It gave a positive Scherer reaction and contained phosphorus in organic combination.

Since the acid could not be crystallized, it was converted into the barium salt. The acid was dissolved in 10 cc. of water and the solution was neutralized with barium hydroxide, after which 20 cc. of alcohol were added. The white amorphous powder which separated was collected, washed with alcohol, and dried *in vacuo*. The product was a white, amorphous powder which weighed 76 mg. The salt was analyzed after it had been dried at 78° *in vacuo* over dehydrite.

*Analysis*—Found, Ba 40.61, 40.60; P 8.99, 9.05

These values correspond to the formula  $C_9H_{16}O_{14}P_2Ba_2$ .

$C_9H_{16}O_{14}P_2Ba_2(684.8)$ . Calculated, Ba 40.13, P 9.05

A barium salt of similar composition was isolated after alkaline saponification of the phosphatide of the human tubercle bacillus (8). It was shown, however, that this organic phosphoric acid on hydrolysis gave mannose (9), and not inosite. The organic phosphoric acid isolated in the present investigation must have contained inosite and it was probably a compound of glycerophosphoric acid and inosite monophosphoric acid.

*Discussion*—The polysaccharides which are combined in the firmly bound lipids in the three types of acid-fast bacteria that have been studied in this Laboratory up to the present time vary in composition with the type of bacteria. The various cleavage



products of the polysaccharides that have been identified are shown in Table III.

It will be noticed that each polysaccharide when hydrolyzed with dilute sulfuric acid yields about 50 per cent of reducing sugars and that the latter have been accounted for by the amounts of mannose, *d*-arabinose, or galactose that have been isolated. So far it has been impossible to identify the non-reducing portion of the polysaccharides. Small amounts of inosite have been isolated from the hydrolysis products of the polysaccharides from the human and avian bound lipids, but they represented only an

TABLE III  
*Cleavage Products of Polysaccharides Contained in Firmly Bound Lipids*

Type of bacillus	Human A-10	Leprosy, Strain 370	Avian
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Total reducing sugar on hydrolysis.....	57.0	50.5	50.1
<i>d</i> -Mannose.....	6.6	None	27.3
<i>d</i> -Arabinose.....	38.7	41.4	19.9*
Pentose as phloroglucide.....	39.5	48.3	30.7
Other pentoses.....	None	7.0	Probably none
<i>d</i> -Galactose.....	12.2	1.0	3.5*
Inosite.....	Small amount	None	Small amount
Precipitin reaction.....	1:1,000,000	1:2,000,000	1:500,000
Glucosamine†.....	Present	None	Present

\* These values are too low because a portion of the material was lost.

† Determined by the method of Palmer, Smyth, and Meyer (10).

insignificant fraction of the non-reducing portion. The polysaccharides from the human and avian bound lipids contained small amounts of nitrogen but the nature of the nitrogen compounds has not been fully established. Only small amounts of glucosamine were indicated by color reactions. Further studies will be necessary to determine the nature of the non-reducing portion of the hydrolyzed polysaccharides as well as the phosphorus and nitrogen compounds which occur in the polysaccharides of the bound lipids of the human and avian tubercle bacilli.

#### IV. *Composition of the Filtrable Portion of the Firmly Bound Lipids*

The filtrable fraction of the lipid which had been precipitated from ether solution with alcohol, as described in Part I, was a

white powder weighing 21.5 gm., m.p. 60–90°. A portion of this substance was saponified and the cleavage products were examined.

For saponification 10 gm. of the substance were dissolved in 100 cc. of benzene and 30 cc. of absolute alcohol containing 2.0 gm. of potassium hydroxide were added. The solution was perfectly clear at first, but on being heated to boiling on a water bath a precipitate began to separate. The solution was refluxed for 6½ hours and allowed to cool to room temperature, after which the supernatant was decanted. The insoluble material which consisted of water-soluble carbohydrate formed a hard crust on the bottom of the flask and this was rinsed several times with warm benzene.

TABLE IV  
*Cleavage Products on Saponification of Filtrable Lipid*

	<i>gm.</i>	<i>per cent</i>
Hydroxy fatty acids.....	6.9	69.0
Lower fatty acids.....	0.37	3.7
Neutral material.....	0.82	8.2
Crude polysaccharide.....	1.50	15.0
Total recovered material.....	9.59	95.9

The alkaline benzene-alcohol solution was refluxed on a water bath for 15 hours, but there was no further precipitation of carbohydrate. The ether-soluble constituents were isolated and fractionated as described for the corresponding material obtained from the unfiltrable lipid. The carbohydrate was isolated by precipitation with basic lead acetate in the usual manner. The products thus obtained are summarized in Table IV.

Owing to the small amount of lower fatty acids this material was not further examined.

A search was made for glycerol among the cleavage products, but none was found. The polysaccharide was not fully examined because the amount obtained was too small to permit of a complete analysis. It was determined, however, that the substance gave the same pentose color reactions as the polysaccharide isolated from the unfiltrable lipid.

*The Neutral Material. Isolation of d-Eicosanol-2*—The neutral material consisted principally of *d*-eicosanol-2. The substance

was recrystallized from ethyl acetate and gave colorless prismatic needles which melted at  $62^{\circ}$  and gave no depression of the melting point when mixed with a previously isolated sample of *d*-eicosanol-2.

*Rotation*—0.3789 gm. of substance dissolved in ether and diluted to 10 cc. gave, in a 1 dm. tube, a reading of  $+0.26^{\circ}$ ; hence  $[\alpha]_D^{26} = +6.86^{\circ}$ .

*Analysis*— $C_{20}H_{42}O$  (298).    Calculated.    C 80.53, H 14.09  
   Found.        " 80.43, " 14.49

*The Hydroxy Fatty Acids. Isolation of Avian  $\gamma$ -Mycolic Acid*—The hydroxy acids, as shown in Table IV, represented the chief constituent of the filtrable lipid. For purification the substance, 6.9 gm., was dissolved in 150 cc. of warm ether and the solution was treated with norit and filtered. The colorless filtrate was concentrated to about 50 cc. and mixed with an equal volume of acetone, whereupon a voluminous precipitate separated which was filtered off and washed with acetone. The substance was reprecipitated four times in the same manner. The product thus obtained, Fraction I, was a snow-white amorphous powder, which under the microscope appeared as fine, colorless, globular particles. The substance was only slightly soluble in cold ether, but it dissolved in warm ether. It was practically insoluble in cold acetone and it was more soluble in a mixture of warm ether and acetone than in pure ether.

The mother liquors were concentrated to a volume of about 50 cc. and allowed to cool. The substance that separated was reprecipitated from a mixture of ether and acetone and was obtained as a snow-white amorphous powder which was designated Fraction II. In solubility and properties this substance appeared to be identical with Fraction I. The data are summarized in Table V.

Both fractions were tested for methoxyl by the Zeisel method, but no volatile iodide was obtained.

The results show that the two fractions were very similar in properties and in composition. The substance will be designated by the name avian  $\gamma$ -mycolic acid.

The equivalent weights were determined by titration and are undoubtedly lower than the true molecular weight. It is very probable that we are dealing with a dicarboxylic acid.

*The Methyl Esters*—The methyl esters of Fractions I and II of the avian  $\gamma$ -mycolic acid were prepared by treatment with diazomethane in ether solution. The solvent was evaporated and the residue was dissolved in ether and precipitated by the addition of acetone. Both ester fractions were obtained as white amorphous powders and both melted at 48–49°. The hydroxyl values given in Table V for the acid fractions were determined on the esters by the method of Stodola (11).

*Acetyl Derivative of Avian  $\gamma$ -Mycolic Acid*—A sample of the acid, Fraction I, 0.51 gm., was dissolved in 10 cc. of pyridine and 2.0 cc. of acetic anhydride were added. After the solution had stood for 2 days at room temperature, it was poured into dilute hydrochloric acid. The white amorphous precipitate that separated was filtered off, washed thoroughly with water, and dried *in vacuo*. The white powder weighed 0.53 gm. The substance

TABLE V  
*Properties and Composition of Avian  $\gamma$ -Mycolic Acid*

Fraction No.	Weight	M.p.	$[\alpha]_D$ in $\text{CHCl}_3$	Mol. wt.	Iodine No.	Hydroxyl value	Carbon	Hydrogen
	gm.	°C.	degrees			per cent	per cent	per cent
I	4.8	65	+5.3	778	4.1	1.15	80.89	13.24
II	1.5	65	+5.7	648	9.0	1.23	79.47	13.39

was dissolved in 20 cc. of ether and the solution was mixed with an equal volume of acetone. On cooling the solution in ice water, a white globular powder separated which was collected, washed, and dried. The product weighed 0.4 gm. and melted at 43–44°. The acetylated product was much more soluble in ether than was the original acid.

*Titration*—0.3747 gm. of substance dissolved in 50 cc. of ether required 4.26 cc. of 0.08566 N alcoholic KOH. Found, mol. wt. 1026.

*Analysis*—Found, C 80.51, H 13.34.

#### V. *Pyrolysis of the Hydroxy Acids of the Filtrable Portion of the Firmly Bound Lipids*

It appears to be a general property of the hydroxy acids isolated from the acid-fast bacteria to split on pyrolysis, yielding crystalline fatty acids which distil off. The acids thus produced vary,

however, in composition and constitution with different types of bacteria.

It has been found in this Laboratory that mycolic acid obtained from the wax of the human tubercle bacillus yields on pyrolysis normal hexacosanoic acid, which distills off, leaving a non-volatile neutral residue (4). The bovine mycolic acid behaves in a similar manner (12). A hydroxy acid isolated from the firmly bound lipids of the so called leprosy bacillus gave, on pyrolysis, a crystalline acid which appeared to be a branched chain tetracosanoic acid,  $C_{24}H_{48}O_2$ , m.p.  $76-77^\circ$ .

The wax isolated from the avian tubercle bacillus yielded two hydroxy acids (13) named avian  $\alpha$ - and  $\beta$ -mycolic acid, which also split on pyrolysis. The avian  $\alpha$ -mycolic acid gave a branched chain pentacosanoic acid,  $C_{25}H_{50}O_2$ , m.p.  $78-79^\circ$ , while the  $\beta$ -mycolic acid gave normal tetracosanoic acid,  $C_{24}H_{48}O_2$ , m.p.  $83^\circ$  (13).

The hydroxy acid isolated in the present investigation differed in properties from both avian  $\alpha$ - and  $\beta$ -mycolic acids and hence we have named it avian  $\gamma$ -mycolic acid, as stated in Part IV. It appeared of interest to determine what products this new acid would give on pyrolysis.

Since the two fractions of avian  $\gamma$ -mycolic acid were so similar in properties, a mixture containing 1.5 gm. of Fraction I and 0.5 gm. of Fraction II was used in the pyrolysis experiment. The acid contained in a small distilling flask provided with a wide outlet tube was heated in an air bath at a pressure of about 0.1 mm. At a temperature of the air bath of  $270-280^\circ$  there was a rapid distillation of a colorless oil, which immediately crystallized in the cool part of the outlet tube, and the reaction was completed in about 15 minutes. The crystalline distillate weighed 0.36 gm., corresponding to 18 per cent of the hydroxy acid.

The residue in the distilling flask was a hard, faintly yellowish, wax-like material, which was examined, as will be described later.

*Examination of the Distillate*—The crystalline distillate was removed and recrystallized three times from acetone. The product separated in the form of branching feathery crystals similar to the pentacosanoic acid obtained from the avian  $\alpha$ -mycolic acid. The crystals weighed 0.3 gm. and melted at  $80-81^\circ$ , solidified at  $79^\circ$ , and remelted at  $81-82^\circ$ .

The mother liquors were concentrated to about 5 cc. and the small amount of acid which separated in bundles of feathery crystals was collected, washed, and dried. This fraction melted at 80–81°, solidified at 79°, and remelted at 81–82°.

The fact that both the first and second crop of crystals melted at the same temperature would indicate that the distilled acid was homogeneous.

In a mixed melting point with a sample of the pentacosanoic acid, m.p. 78–79°, obtained from the avian  $\alpha$ -mycolic acid, there was a depression of 2°. Evidently, therefore, the two acids are not identical.

*Titration*—0.1260 gm. and 0.1312 gm. of substance dissolved in a mixture of benzene and neutral alcohol required 3.50 cc. and

TABLE VI  
*Properties and Composition of Non-Volatile Fractions of  
Avian  $\gamma$ -Mycolic Acid*

Fraction No.	Iodine No.	Neutral equivalent	Carbon	Hydrogen	M.p. of ester	Hydroxyl value of ester
			<i>per cent</i>	<i>per cent</i>	<i>°C.</i>	<i>per cent</i>
I	19	1385	82.26	13.21	48–49	0.93
II	16	935	82.50	13.57	41–44	None

3.65 cc. of 0.09694 N alcoholic KOH. Found, mol. wt. 371 and 370.

*Analysis*—Average of three analyses

$C_{24}H_{48}O_2$ (368). Calculated. C 78.26, H 13.04

Found. " 78.08, " 13.03

The analytical values found are in close agreement with the calculated composition of a tetracosanoic acid. The peculiar crystal form of the acid, however, would suggest that it possesses a branched chain structure.

*Examination of the Non-Volatile Residue*—The residue in the distilling flask was dissolved in about 20 cc. of warm ether and as the solution was cooled fine globular particles separated. The precipitate was filtered off, washed with cold ether, and dried *in vacuo*. The product, Fraction I, was a white, voluminous, amorphous powder which weighed 0.87 gm. On heating in a capillary tube, the substance sintered at 48° and melted at 58°.

The filtrate from the above product was diluted with an equal volume of acetone and cooled in ice water. The voluminous white precipitate, Fraction II, which separated was collected, washed with acetone, and dried *in vacuo*. The snow-white powder weighed 0.67 gm. and melted at 49–50°.

Both fractions were unsaturated and they showed acid properties when titrated in ethereal solution with alcoholic potassium hydroxide. The methyl esters were prepared by means of diazomethane in ethereal solution and the hydroxyl value of the esters was determined by the method of Stodola (11). Fraction I apparently contained one hydroxyl group to one carboxyl, whereas Fraction II gave no hydroxyl value. Since both fractions were acid in reaction, they must have contained at least one carboxyl group. Evidently, therefore, the original  $\gamma$ -mycolic acid must have contained at least two carboxyl groups.

No claim to purity can be made for the two fractions of the non-volatile material, but certain analytical determinations were made and the results are recorded in Table VI.

#### SUMMARY

1. Avian tubercle bacilli after thorough extraction with neutral solvents yielded 10.8 per cent of firmly bound lipids on treatment with dilute hydrochloric acid and extraction with ether and chloroform.

2. The firmly bound lipids were separated into filtrable and un-filtrable fractions by forcing a chloroform solution of the mixture through a Chamberland filter.

3. The unfiltrable lipid gave on saponification 64 per cent of ether-soluble constituents and 31 per cent of a water-soluble polysaccharide. (a) The ether-soluble components consisted of 76.2 per cent hydroxy acids of high molecular weight and unknown constitution, 5.6 per cent of lower fatty acids, and 15.5 per cent of neutral material. (b) The neutral material consisted mainly of *d*-eicosanol-2,  $\text{CH}_3(\text{CH}_2)_{17}\text{CHOHCH}_3$ . (c) The polysaccharide gave on hydrolysis 50.1 per cent of reducing sugars consisting of mannose, *d*-arabinose, and galactose, together with traces of glucosamine and inositol.

4. The solid portion of the filtrable lipid gave on saponification 80.9 per cent of ether-soluble components and 15.0 per cent of

polysaccharide. (a) The ether-soluble components consisted of 69.0 per cent of hydroxy acids, 3.7 per cent of lower fatty acids, and 8.2 per cent of neutral material. (b) The neutral material consisted mainly of *d*-eicosanol-2. (c) The hydroxy acid, named avian  $\gamma$ -mycolic acid, was split on heating to 270–280° under reduced pressure, and gave a crystalline acid which distilled off, leaving an unsaturated non-volatile residue. (d) The acid liberated on pyrolysis corresponded in composition to a tetracosanoic acid,  $C_{24}H_{48}O_2$ , but it differed in crystal form from the ordinary straight chain fatty acids.

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## SODIUM AND POTASSIUM IN FROG MUSCLE\*

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The potassium content of muscle fibers depends, in part, upon the potassium concentration of the external medium (Fenn and Cobb, 1934). In tissues intact in the body, it has been shown that sodium enters the fibers as potassium leaves, either because of low potassium in the blood (Heppel, 1939, 1940) or because of activity or fatigue (Fenn, 1938). Such findings suggest that the normal accumulation of potassium is due to some type of diffusion equilibrium which changes as the physiological condition of the cell changes and which probably is influenced by several factors or mechanisms. If in isolated muscle such an equilibrium exists, it should be possible to demonstrate its presence first, by showing that a simple relationship exists between sodium and potassium in muscle fibers (the sum of the concentrations of the two elements should be approximately constant) and second, by the ability of the fibers (after they have lost potassium, because of the low concentration outside) to reconcentrate potassium when the external concentration is raised. The experiments reported here show that potassium loss from isolated frog muscles is reversible and that changes in concentration of this element in the cells are accompanied by compensatory changes in sodium.

### *Methods*

Sartorii from common laboratory frogs (mostly *Rana pipiens*) were used in all cases. These were handled essentially as described by Fenn and Cobb (1934). Muscles were removed with as little injury as possible and soaked for varying periods of time in 50 to 100 times their weight of solution. Two modifications of normal Ringer's solution were made up. One contained, besides the usual

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constituents, 0.01 N KCl (K-Ringer's solution); the other was made up without potassium (K-free Ringer's solution). The total chloride content in all cases was 0.124 N.

As a rule, each muscle was analyzed for both sodium and potassium. The tissues were ashed and the residues put into acid solution and made up to 5 cc. Aliquots were taken for the separate analyses. Variations in sodium and potassium contents of sartorius muscles were within the ranges usually reported (see Fenn (1936)). It was found impractical to determine chloride on the same muscle used for either sodium or potassium analysis and hence the figures correlating chloride space with potassium content were arrived at by subjecting paired muscles to exactly the same experimental procedure and then analyzing one for chloride, the other for potassium and sodium. Other figures for chloride compared with sodium and potassium are also reported; these were obtained by averaging four or more determinations of each element on muscles from different animals which were carried through the same experimental procedures at the same time.

Muscles were routinely tested for irritability by single condenser shocks. Since no particular relationship was observed between loss of potassium and loss of irritability, the results will not be reported in detail.

Potassium was determined by the chloroplatinate method used in other investigations (Steinbach, 1937). Sodium was precipitated by the method of Salit (1932)<sup>1</sup> and the rest of the determination carried through according to the method of Ball and Sadusk (1936). Some difficulties were encountered with the washing of the uranyl zinc sodium acetate precipitate, apparently owing to a change in the salt used to saturate the acetic acid wash fluid. These difficulties were overcome by saturating the wash fluid with freshly prepared triple acetate several hours before each day's run.

Chloride was determined by a method slightly modified from that of Patterson (Peters and Van Slyke, 1932). All methods were standardized against standard solutions and rechecked frequently.

<sup>1</sup> In accordance with Ball and Sadusk and others it was found essential to stir the solution rapidly during precipitation of the Na. The simplest and most effective way found for stirring in a 15 cc. centrifuge tube was to use the high frequency, low amplitude vibrations from an inexpensive electric shaver, transmitted through a slender glass stirring rod.

*Results*

Fig. 1 summarizes the most important findings of these investigations. Although there is some variability, it is clear that there is a proportional gain of sodium as potassium is lost. The chloride

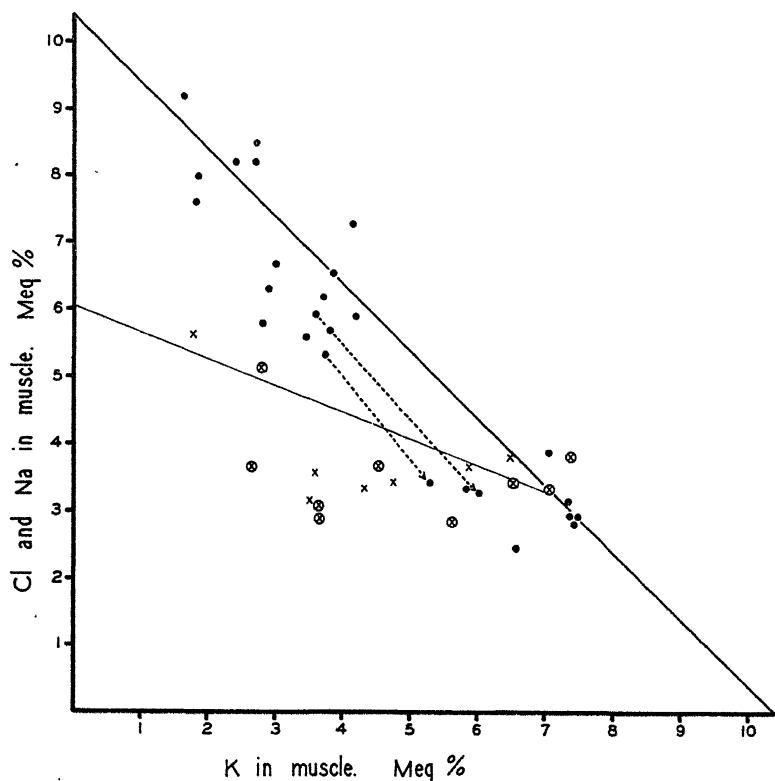


FIG. 1. Na and Cl plotted against K in muscle tissue. The concentrations are expressed in milliequivalents per 100 gm. of the final wet weight of the tissue. The dots represent Na and K, the crosses, Cl and K, each determined by analysis of one of a pair of sartorii. The crosses in circles represent Cl and K, each point representing average figures from four or more analyses of muscles from different frogs but treated in the same way at the same times. The figures are not corrected for extracellular space. The light line represents the chloride values that would be expected if all the potassium loss were due to increased chloride space. The points connected by the dotted lines illustrate reversal of the potassium loss and sodium gain (see the upper part of Table I and also the description in the text).

space measurements show that this is an exchange phenomenon as long as the potassium content remains above about 3 milliequivalents per cent of the final wet weight. When more potassium is lost, chloride is apt to enter along with the sodium, presumably indicating the destruction of cells and the abolition of the normal physiological exchange reaction. The fact that the experimental points fall fairly well along the same straight line regardless of chloride space changes is to be expected, since the cells are

TABLE I  
*K and Na Exchange in Frog Muscle*

The results are given in milliequivalents per cent of the final wet weight of tissue.

Frog o.	Muscle No.*	Na	K	Cl	Change
1	1	5.95	3.60	2.82	Na -2.67, K
	2	3.28	6.05	2.94	+2.45, Cl
2	1	5.35	3.75	2.97	none
	2	3.42	5.30	2.78	Na -1.93, K
Pooled analyses†	1				+1.55, Cl
					none
	2	5.04	3.44	3.79	Na -1.00, K
		(4)	(8)	(8)	+1.55, Cl
	2	4.04	4.99	4.10	+0.31
		(4)	(8)	(8)	

\* All muscles were soaked overnight (17 hours) in K-free Ringer's solution; Muscles 1 were then removed for analysis and Muscles 2 were placed in K-Ringer's solution (Ringer's fluid made up 0.01 N in potassium) for 6 to 8 hours and then analyzed.

† The figures in parentheses give the number of separate analyses making up the average figure. The figures are not corrected for extracellular space.

immersed in isotonic NaCl solution and either an ion exchange or penetration of the complete salt would show about the same gain of sodium as potassium is lost.

Experiments were carried out to show that the ion exchange is reversible. Paired muscles were soaked overnight in K-free Ringer's solution. One member of each pair was then taken for analysis; the other muscle was transferred to K-Ringer's solution for 6 to 8 hours and then analyzed. Control pairs of muscles from other frogs were treated the same way and analyzed for chloride.

The upper part of Table I gives, as representative results, such a series in which the chloride space showed no appreciable change. The same results are shown in Fig. 1 as the points connected by dotted lines. It is clear that a considerable part of the potassium lost to K-free Ringer's solution is regained upon immersion in K-Ringer's solution and that the sodium exchange has been opposite and nearly equal.

The lower part of Table I gives average figures for all of the experiments carried out to test the reversibility of the potassium loss. These are not reported in detail, since the control analyses for chloride showed several cases in which the chloride space increased markedly and hence the figures lose their comparative

TABLE II  
*Average K Contents of Frog Sartorii Soaked in K-Free Ringer's Solution*

	No. of analyses	K in tissue
<i>hrs.</i>		<i>m.eq. per cent final wet weight</i>
2	4	7.10
5	2	6.65
17	10	3.45
18	4	3.35
19	7	3.00
24	4	2.73
27	2	2.87

validity to a certain extent. The averages, however, show the same general type of change, only to a less marked extent. This would indicate that even when some of the fibers of a muscle die, the surviving cells still possess the power to accumulate potassium. The same results also could be interpreted as showing that the penetration of chloride into the cells is irreversible.

In a general way, potassium loss to K-free Ringer's solution is a function of time (Table II). However, considerable variability was noted both with respect to the rate of potassium loss and to the rate at which muscle fibers died, as indicated by an increase in the chloride content. The cause of this variation was not ascertained, but it should be emphasized that the length of time of treatment of a given tissue is of little value in accurately estimating ion changes in that tissue.

Several experiments were carried out in which muscles were soaked overnight in K-Ringer's solution. These experiments confirm Fenn's results showing that muscles neither gain nor lose K to a Ringer's fluid approximately 0.01 N in potassium.

#### DISCUSSION

From these results it is clear that potassium and sodium in the cells of isolated frog muscle are in some sort of equilibrium with the ions of the external medium. The experiments give no information about relative rates of penetration of sodium or potassium ions but they do rule out the possibility that a simple sieve mechanism can account for the concentration of potassium in normal muscle cells. It seems highly probable that some mechanism like that proposed by Teorell (1935) would be operative, particularly since potassium chloride, of all the naturally occurring electrolytes, is most nearly "indifferent." Since the ionic ratios are not consistent with this scheme, however, there must be other important factors. One of these, which has received little experimental attention, is the possibility that the activities of the sodium and potassium compounds formed within the cell may be quite different. If, for example, the apparent dissociation of some organic potassium compound within the cell was less than that of the corresponding sodium compound and if the organic anion was indiffusible, then the distribution ratios of total sodium and potassium within and without probably would be altered in the correct direction; that is, toward a high cellular content of potassium.

Conway and Boyle (1939) have recently published a short note on the mechanism of potassium accumulation. In the absence of a more extended presentation of their theory, it suffices to point out here that their final conclusion, that the potassium concentration inside the cell is equal to half the total external osmotic concentration, obviously does not hold for these observations.

#### SUMMARY

As potassium is soaked out of frog muscle by treatment with K-free Ringer's solution, sodium enters in exchange. This exchange is reversible as long as the muscles do not lose more than about half of their original potassium content and provided that the chloride content of the tissue remains about the same.

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## PREPARATION OF *d*(-)-GLUTAMIC ACID FROM *dl*-GLUTAMIC ACID BY ENZYMATIC RESOLUTION

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It has previously been shown (1) that papain as well as other proteinases can perform the enzymatic synthesis of CO—NH linkages. Thus, in the presence of HCN-papain, carbobenzoxyglycine and aniline were combined to form carbobenzoxyglycine anilide. When a derivative of an asymmetric amino acid was employed, only the derivative of the *l*-amino acid participated in the synthesis. The asymmetric course of this reaction provides the basis for a general method for the resolution of racemic amino acids. In this note the preparation of the "unnatural" *d*(-)-glutamic acid is described. This amino acid has been employed for the synthesis of peptides required in studies on the antipodal specificity of proteolytic enzymes of normal and pathological tissues.

Carbobenzoxy-*dl*-glutamic acid was treated with aniline in the presence of papain-cysteine. The filtrate from the carbobenzoxy-*l*-glutamic acid anilide usually yielded a mixture of carbobenzoxy-*d*- and *l*-glutamic acids in the proportion of 4:1. On hydrogenation and conversion of the glutamic acids to the hydrochlorides, it was possible to obtain, after a few recrystallizations (2), pure *d*(-)-glutamic acid.

Resolutions of *dl*-glutamic acid have been described by Schulze and Bosshard (3), Fischer (4), and Ehrlich (5). Recently Ivanovics and Bruckner (6) have isolated *d*(-)-glutamic acid from the capsular substance of bacteria of the *mesentericus* group.

### EXPERIMENTAL

*dl*-Glutamic Acid Hydrochloride—This substance was prepared according to the method described in an earlier paper (7).

*Carbobenzoxy-dl-Glutamic Acid*—This compound was prepared as described for the *l* form (8). 65 gm. of *dl*-glutamic acid hydrochloride yielded 81 gm. of carbobenzoxy-*dl*-glutamic acid. M.p., 119°.

$C_{13}H_{15}O_5N$ .	Calculated.	C 55.5, H 5.4, N 5.0
281.1	Found.	" 55.4, " 5.4, " 5.2

*Resolution of Carbobenzoxy-dl-Glutamic Acid with Papain-Cysteine*—70 gm. of carbobenzoxy-*dl*-glutamic acid were dissolved in 135 cc. of 2 N NaOH and added to 47 gm. of aniline. A solution of 3 gm. of cysteine hydrochloride in 100 cc. was added, followed by the enzyme solution containing 8 gm. of purified papain (9) in 200 cc. of water. 200 cc. of 0.2 M citrate buffer (pH 5.0) were added and the reaction mixture was made up to 1 liter. Crystallization of the carbobenzoxy-*l*-glutamic acid anilide began almost instantly. The reaction mixture was left at 40° for 2 days. The crystalline precipitate was filtered off, and the filter cake resuspended in 600 cc. of water and refiltered. All the filtrates and washings were combined and the clear solution was concentrated to about 200 cc. Concentrated hydrochloric acid was added (Congo red acidity) and the resulting syrup extracted three times with ethyl acetate. The ethyl acetate layer was washed with water, dried, and concentrated to a syrup. Petroleum ether was added; and, after the material had stood overnight in the cold, the syrup crystallized. The air-dried material weighed 38 gm.  $[\alpha]_D^{23} = +4.1^\circ$  (8.4 per cent in glacial acetic acid). The specific rotation of carbobenzoxy-*l*-glutamic acid is  $-7.1^\circ$  (8).

*d-Glutamic Acid Hydrochloride*—35 gm. of the above mixture of carbobenzoxy-*d*- and *l*-glutamic acids were dissolved in 100 cc. of methyl alcohol and hydrogenated with palladium black as the catalyst. The hydrogenation required 8 to 10 hours and the glutamic acid separated out. Water was added and the catalyst was filtered off and washed with hot water. The combined filtrates were evaporated down, yielding a crystalline residue. This material was dissolved in 70 cc. of 20 per cent hydrochloric acid, and dry HCl passed through the solution. The crystals that separated out on standing in the ice box were collected the next day and dried *in vacuo* over KOH. Yield, 16 gm.  $[\alpha]_D^{23} = -23.0^\circ$  (3.4 per cent in 10 per cent hydrochloric acid).

Two recrystallizations from 20 per cent hydrochloric acid raised the rotation to the correct value. Yield, 11.8 gm., or 46 per cent of the theory (based on the quantity of *dl*-glutamic acid hydrochloride employed).  $[\alpha]_D^{23} = -32.0^\circ$  (3.6 per cent in 10 per cent hydrochloric acid).

$C_6H_9O_4N \cdot HCl$	Calculated.	C 32.7, H 5.5, N 7.6
183.6	Found.	" 32.9, " 5.4, " 7.5

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## THE INERTIA OF HIGHLY UNSATURATED FATTY ACIDS IN THE ANIMAL, INVESTIGATED WITH DEUTERIUM\*

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There are two general methods of investigating the ability of animals to synthesize fatty acids. The first of these involves balance studies on diets restricted with respect to fats or individual fatty acids. By this procedure it has been found that animals can synthesize all "natural" fatty acids except those containing two and three double bonds, such as linoleic and linolenic acids. As these unsaturated acids are required in the diet in small amounts, their function has frequently been considered to be similar to those of the vitamins. Their absence from the diet leads to a characteristic deficiency syndrome (1) and to their slow disappearance from the animal fat (2).

The second method of investigation involves the use of isotopes. If the body fluids of animals are artificially enriched with deuterium oxide, the synthesis of fatty acids from smaller units will necessarily result in the formation of fatty acids containing stably bound deuterium (not removable by treatment with aqueous acid or alkali). It has been found (3, 4) that the content of stably bound deuterium of the fatty acids of mice kept under such experimental conditions increases with time even when the animal is in a steady state with respect to fat content and fat composition. The results indicated a simultaneous and equivalent synthesis and destruction of fatty acids in normal animals.

This interpretation presupposes the absence of any biological

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mechanism which permits mere physical exchange of carbon-bound hydrogen when no chemical reaction occurs. In only two cases has it as yet been found possible definitely to exclude the occurrence of such a mechanism whereby carbon-bound hydrogen is merely labilized. It has been found (5) that the amino acid lysine, in contrast to other amino acids, does not take up deuterium from the body fluids of animals given heavy water to drink, indicating (a) that lysine is not formed in the animal from other substances, and (b) that no labilization of carbon-bound hydrogen of this substance occurs *in vivo*. Analogous findings were obtained on fatty acids in the yolk of hen's eggs<sup>1</sup> (3).

For the investigation of unsaturated acids, we have kept mice in two series of experiments on a diet so low in fat that deposition of dietary fatty acids, especially unsaturated ones, must have been negligible. The deuterium content of the body fluids was raised, as in earlier experiments (3-5), by giving the animals dilute heavy water to drink. The animals were kept under these conditions for 7 and 15 days, when they were killed. From the body fat, highly unsaturated fatty acids were isolated as their difficultly soluble bromides. The total amounts found were low, as was to be expected from the relatively long period of fat starvation, and only one individual highly unsaturated fatty acid could, therefore, be isolated from each sample of fat. In the first series of experiments, the tetrabromide of  $\alpha$ -linoleic acid was isolated; in the

<sup>1</sup> The statement regarding the lack of fat formation in the developing egg may need qualification. While no fatty acid synthesis occurs in the yolk, a very limited synthesis has now been observed to occur in the embryo. In a recent study, to be continued, we have repeated the earlier experiments (3) by injecting 0.8 cc. of  $D_2O$  into a fertilized chicken egg which was then incubated for 17 days. The water of the egg contained  $1.62 \pm 0.02$ , the fatty acids from the yolk  $0.01 \pm 0.02$ , but those from the embryo  $0.14 \pm 0.02$  atom per cent deuterium respectively. The negative results in the previous experiments are due to the fact that the embryo and yolk were worked up together. Since the yolk contains about 8 times as much total fatty acid as the embryo, the deuterium of the latter was diluted with the normal hydrogen of the former to a value (0.01 atom per cent) so low as to be within the experimental error of our method. This finding of a small amount of deuterium in the fatty acid of the embryo is of interest in connection with the results obtained with radioactive phosphorus by Hevesy and Hahn (6) who found phospholipid synthesis to occur in the tissues of the embryo but none in the yolk.

second, a hexabromide melting sharply at 139–140° was obtained. The latter material is not identical with the insoluble hexabromide, m.p. 179°, of linolenic acid from linseed oil. We have not succeeded in isolating any tetrabromides from these animals. Only a small quantity of linoleic acid could have been present.

*None of the samples of highly unsaturated fatty acids contained any deuterium above the experimental error of the analysis.* On the other hand, the saturated acids, as well as the total unsaturated acids consisting mainly of oleic acid, contained considerable quantities of stably bound deuterium.

The absence of deuterium in the highly unsaturated compounds definitely indicates that: (1) In contrast to stearic, palmitic, and oleic acids, neither of the two highly unsaturated acids had been biochemically synthesized from smaller units. (2) Whereas oleic acid and stearic acid are mutually interconvertible by a process of hydrogenation and dehydrogenation in the animal body (7, 8), the more highly unsaturated fatty acids cannot have been derived from stearic acid by dehydrogenation. (3) No mere physical exchange of carbon-bound hydrogen occurs in these highly unsaturated fatty acids, not even in the course of their esterification with glycerol (a reaction in which they are known to participate in the animal). The first two conclusions are in agreement with the findings of earlier investigators on the indispensability of these acids. The third may be taken as additional evidence in support of the view that no fatty acid undergoes simple exchange reactions in the animal. The biological introduction, in one and the same animal, of deuterium into some species of fatty acids but not into others can scarcely be explained in any other way than that the former have partaken in chemical reactions involving carbon-bound hydrogen atoms but the latter have not.

#### EXPERIMENTAL

*Experiment A*—Twenty male mice of 22 gm. average weight were placed on a diet consisting of dried whole wheat bread exhaustively extracted with ether. For drink, they were given 4 per cent heavy water. After 15 days they were killed. A sample of water distilled from the bodies contained 2.96 atom per cent. The total fatty acids of the bodies were isolated, and the saturated and unsaturated fractions were separated by the lead salt method.



The total fatty acids contained 0.62, the saturated fraction 0.76, and the unsaturated fraction 0.49 atom per cent deuterium respectively.

*Isolation of Tetrabromide of  $\alpha$ -Linoleic Acid*—To a solution of 12.7 gm. of unsaturated fatty acids in petroleum ether (purified with concentrated  $\text{H}_2\text{SO}_4$ ) was added a solution of bromine in petroleum ether until a faint red color persisted. The precipitate formed was filtered off and extracted with petroleum ether by shaking for several hours in a glass-stoppered bottle. The undissolved material (1.98 gm.) was completely soluble in ether, chloroform, and hot ethanol. It was recrystallized from hot ligroin from which it separated in long needles of melting point  $114\text{--}115^\circ$  unchanged on admixture with an authentic sample of the tetrabromide of  $\alpha$ -linoleic acid prepared from poppy oil according to Rollett (9).

*Analysis*— $\text{C}_{18}\text{H}_{32}\text{Br}_4\text{O}_2$ . Calculated, Br 53.31; found, Br 53.28

For the deuterium analysis (10), 400 mg. were burned after being mixed with powdered silver oxide. The resulting water contained only traces of bromine which were removed by treatment for 1 day with copper metal. The purified water contained  $0.02 \pm 0.02$  atom per cent deuterium.

*Experiment B*—Forty male mice of 20 to 22 gm. each were injected subcutaneously with 0.5 cc. of concentrated  $\text{D}_2\text{O}$  in order to raise the deuterium content of the body fluids to about 2.5 atom per cent, and were then given 4 per cent heavy water instead of ordinary drinking water. After 7 days they were killed and the internal organs (liver, spleen, lungs, kidneys, and intestinal tract) were removed. Samples of tissue water were distilled from groups of ten animals and from the combined organs of groups of twenty animals. The deuterium analyses of these are given in Table I.

In order to prevent oxidation of the highly unsaturated fatty acids, each group of ten carcasses was placed in 300 cc. of a solution containing 25 per cent of KOH, 25 per cent of methanol, and 50 per cent of  $\text{H}_2\text{O}$ . These mixtures were kept for 2 days at  $30^\circ$ , when all material except the skeletons was dissolved, filtered through glass wool, acidified with  $\text{H}_2\text{SO}_4$ , and four times extracted with pure petroleum ether. The extracts were concentrated to a

small volume and again treated with methanolic KOH. The unsaponifiable fraction was removed by exhaustive extraction with petroleum ether, and the fatty acids were obtained after acidification and extraction with petroleum ether. The total amount of fatty acid was determined in an aliquot and found to be 44.3 gm. A portion of this same aliquot was found to contain  $0.41 \pm 0.02$  atom per cent deuterium. Another portion was separated into saturated and unsaturated fractions. The saturated fraction contained  $0.58 \pm 0.02$  and the unsaturated fraction  $0.34 \pm 0.02$  atom per cent deuterium, respectively.

*Isolation of Octadecatrienoic Acid Hexabromides*—To the remainder of the total carcass fatty acids (30.8 gm.) in petroleum ether was added bromine, as described above. 3.63 gm. of crude bro-

TABLE I  
*Contents of Deuterium in Body Fluids*

Group No.	Deuterium in water	
	From carcass	From internal organs
	<i>atom per cent</i>	<i>atom per cent</i>
I	2.39	2.37
II	2.31	
III	2.24	2.19
IV	2.26	

mides were obtained, which were shaken three times for 3 hours each with petroleum ether. The undissolved material was extracted with hot benzene to separate tetra- and hexabromides from the insoluble octa- and decabromides. The benzene was evaporated *in vacuo*, and the crystallized residue twice recrystallized from hot ethanol. The substance melted sharply at 139–140°.

*Analysis*— $C_{18}H_{30}O_2Br_6$ . Calculated, Br 63.29; found, Br 63.24

As the amount of material was small, it was combined with a sample obtained from the alcoholic mother liquor, which might have contained some tetrabromides. The combined material contained  $0.01 \pm 0.02$  atom per cent deuterium.

## SUMMARY

1. The question of the formation of doubly and triply unsaturated fatty acids was studied with the aid of deuterium in mice on a fat-low diet of bread and heavy water.

2. In agreement with earlier experiments, the saturated as well as the total unsaturated fatty acids isolated from these animals contained considerable amounts of stably bound deuterium, the saturated fraction more than the unsaturated one.

3. The unsaturated fractions yielded  $\alpha$ -linoleic acid as the tetrabromide and a triply unsaturated acid as the hexabromide. None of the samples contained an amount of deuterium above the limit of the error of analysis. These higher unsaturated acids, in contrast to the other fatty acids of the same animals, had therefore not been biochemically synthesized, but must have been derived directly from the diet.

4. The finding is new confirmation that hydrogen of  $-\text{CH}_3$ ,  $-\text{CH}_2-$ , and  $=\text{CH}-$  groups in fatty acids is not exchangeable with the hydrogen of the water of the body fluids in which they are dissolved or suspended. Introduction of hydrogen from body fluids occurs only in the course of chemical reactions.

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# THE RATE OF FORMATION OF STEARIC AND PALMITIC ACIDS IN NORMAL MICE\*

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The appearance of stably bound deuterium in fatty acids of animals given heavy water to drink can be taken as an indicator of fat formation (1, 2). All fatty acids hitherto investigated, with the exception of the doubly and triply unsaturated acids (3), take part in a continuous process of synthesis and destruction. By determining the rate of "uptake" of deuterium into the fatty acids of different organs of animals on a fat-free diet, it is possible to obtain an indication as to the relative "activity" of various organs in regard to fatty acid formation. The faster the process of fat formation in a particular organ, the faster the "uptake" of deuterium into its fatty acids.

*Site of Fatty Acid Formation*—The heavy water content of the body fluids of mice was artificially raised to 2.4 to 4.4 per cent by methods previously described. The mice were kept on a diet low in fat for 2, 3, 6, and 15 days respectively, when they were killed. Fatty acids were isolated from the fat tissues, liver, intestinal wall, and kidneys. Except for the material from the latter organ, the acids were separated into the saturated and unsaturated fractions and their deuterium content determined (Table I).

If formation of fatty acids had occurred mainly in one organ, the deuterium content of its acids should be higher than in those of any other organ. This, however, is true only in experiments of short duration. If the experiments be carried out over a longer

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period, the fatty acids formed in one organ may be equally distributed over all other organs, which then would have the same high deuterium content.

In all experiments the deuterium content of the fatty acids from the liver was higher than in those from the other organs. The liver is thus the most "active" organ. As it is generally accepted that fats from the liver may be transported to the fat tissues and possibly also to other organs, we cannot exclude the possibility that the deuterio fatty acids found in the other organs had their origin in the liver. Of the four organ fats investigated, the deuterium content in that from the depots was lowest, while those from the intestinal wall and kidney were intermediate between liver and depot.

Our finding is in agreement with the results of Ussing (4) who had given rats heavy water to drink and showed the fatty acids of the liver to have a deuterium content higher than that in the fat depot. The author also suggested that fat formation in the liver is more rapid than in other organs.

*Rate of Synthesis of Stearic Acid Compared with That of Palmitic Acid*—The fats of higher mammals contain mainly palmitic, stearic, and oleic acids. The other acids (palmitoleic acid, highly unsaturated acids, etc.) occur in lower concentrations. If the rates of synthesis and destruction of the various fatty acids, *i.e.* their regeneration,<sup>1</sup> differ, they should take up deuterium (from  $D_2O$  of the body fluids) with different rates. We have isolated the saturated fatty acids from the depot fats of mice, the body fluids of which contained 3.46 atom per cent deuterium for a period of only 2 days (Experiment A, Table I). The saturated fatty acid fraction contained 0.55 atom per cent deuterium. Palmitic and stearic acids isolated from this material by fractional distillation of the methyl esters (6) contained 0.56 and 0.54 atom

<sup>1</sup> In previous papers from this laboratory the term "turnover" has been employed for the designation of the process of simultaneous degradation and synthesis of organic compounds. "Turnover," however, is generally employed for the replacement of a compound by another of the same species from any source, including the diet. All substances in the living organism are "turned over," even inorganic ions. However, only certain compounds are "regenerated." Hevesy (5) has employed the word rejuvenation for the replacement of part of a molecule; *i.e.*, for phosphate in phospholipid by other phosphates. "Rejuvenation" is thus included in the concept of "regeneration."

per cent respectively; *i.e.*, the same concentration within the error of the analytical procedure. Similar results had been obtained in earlier experiments from this laboratory (2) when mice were given heavy water to drink for periods of 5 and 37 days. Both palmitic and stearic acids isolated from the bodies of the animals had the same deuterium content as that of the total saturated

TABLE I

*Deuterium Content in Fatty Acids from Mice Given Heavy Water to Drink*

Experiment No.	No. of animals	Duration of experiment	Deuterium in body fluids	Deuterium content (atom per cent) in fatty acids of							
				Depot			Liver		Intestinal wall		Kidney
				Total acids	Saturated acids	Unsaturated acids	Saturated acids	Unsaturated acids	Saturated acids	Unsaturated acids	
		days	atom per cent								
A	20	2	3.46	0.36	0.55	0.26	1.35	0.61	0.72	0.35	0.44
B	10	3	4.43	0.64	0.98	0.50	2.06	1.49	1.41	1.07	0.83
C	10	6	2.40	0.39	0.50	0.32	1.35	1.06	0.82	0.50	0.59
D	20	15	2.96	0.62	0.76	0.49	1.41	0.80	1.02	0.57	0.75

TABLE II

*Deuterium Concentration in Palmitic and Stearic Acids from Mice Given Heavy Water to Drink*

	Deuterium content (atom per cent) in fatty acids		
	Experiment A	Experiment 1*	Experiment 2*
Total saturated acids . . . . .	0.55	0.29	0.58
Palmitic acid . . . . .	0.56	0.30	0.51
Stearic " . . . . .	0.54	0.27	0.63

\* Experiments 1 and 2 correspond to Experiments A and B of the paper by Rittenberg and Schoenheimer (2). The body water of the mice contained 1.70 and 1.62 atom per cent deuterium respectively. The former experiment was of 5 and the latter of 37 days duration.

fraction from which they were secured. As the former experiments were of comparatively long duration, the fatty acids were isolated at a time when their deuterium content was already closer to the maximal deuterium content that can possibly be reached. The new finding taken together with the older ones (Table II) may be taken as indication that both acids are de-

stroyed and resynthesized (regenerated) at the same rate in normal animals.

The fats of normal mice contain several times as much palmitic acid as stearic acid. The finding of equal deuterium concentration in both substances indicates that the same relative proportion of the two acids is regenerated simultaneously. *At the time when half of all palmitic acid molecules are degraded and newly formed, the same has happened to half of the stearic acid molecules.*

While the deuterium content in the major components of the saturated fatty acid fraction is thus the same, the deuterium content of the unsaturated fraction was always found to be lower (1, 2). The same result was obtained in the present study. As the mechanism of biological fatty acid synthesis is presumably the same in principle for the saturated and the unsaturated acids, the finding of different deuterium contents was difficult to explain. The recent finding of the biological inertia of the doubly and triply unsaturated acids (3) may be responsible for this difference. The unsaturated fraction, in contrast to the saturated one, represents a mixture of inert fatty acids (not regenerated and therefore not containing deuterium) with others that are regenerated.

*Rate of Fatty Acid Formation in Liver*—In the previous experiments of this laboratory (2), the rate of "regeneration" of fatty acids of total mice has been studied by measuring during periods up to 96 days the introduction of deuterium from the  $D_2O$  of the body fluids. The maximum deuterium concentration obtained in the saturated fraction was about half of that in the body fluids in which the fatty acids must have been formed. From the shape of the curve it was calculated that the average half life time<sup>2</sup> of the fatty acids in mice kept on diets low in fat was about 5 to 9 days.

<sup>2</sup> It would be a mistake to determine the *complete* replacement of molecules by newly formed ones; *i.e.*, the time necessary for total regeneration. Reactions of such kind follow exponential curves and the time required for complete regeneration is infinite. The values found for total replacement are mainly a measure of the experimental error (physiological variation, analytical error, etc.). The results of Rittenberg and Schoenheimer (2) on the rapid regeneration of fatty acids have been questioned by Kohl (7) who has determined the time of complete disappearance of an unnatural fatty acid (elaidic acid) from the depot of rats after they were overfed with elaidin. He claims the turnover of fats to be much slower than found in this laboratory. The disappearance of elaidic acid from the fats of animals

As the rate of fatty acid regeneration in the liver is much faster than in the fat tissue, the maximum deuterium concentration (*i.e.*, half of that in the body fluids) in the saturated fatty acids of the liver is attained much earlier (Table III). The process of fatty acid regeneration in the liver is so rapid that we have not been able to determine its half time value. In order to establish it, it will be necessary to determine the time when the saturated fatty acids have acquired half their maximum deuterium content; *i.e.*, 25 per cent of the deuterium concentration in the body fluids. In our shortest experiment, of 2 days duration, the value found (38 per cent) was higher. Considerably more than half of the saturated fatty acids was regenerated within 2 days. The finding

TABLE III  
*Deuterium Content in Saturated Fatty Acids of Livers Relative to That in Body Water*

Experiment	Duration of experiment	Deuterium content in saturated acids of livers relative to that in body water*
	<i>days</i>	
A	2	38
B	3	44
C	6	56
D	15	47

$$* \frac{\text{Atom per cent deuterium in acids}}{\text{Atom per cent deuterium in body water}} \times 100.$$

indicates that the half life of the average saturated fatty acid molecule in the liver is very short, probably about 1 day.

The present experiment confirms the earlier findings that during biological fat synthesis the fatty acids acquire a concentration of deuterium about half that in the water of the body fluids in which they are formed. A process of biological fatty acid synthesis must be formulated, in which about 1 out of every 2 hydrogen atoms in the resulting acid is derived from the aqueous medium,

is an irreversible process, certainly different from that of regeneration of physiological molecules followed with deuterium. It is, however, interesting that, on the basis of Kohl's curve, the disappearance of elaidic acid is exactly the same as that of the true regeneration of depot fats, both having a half period of about 8 days.



while the other half had stayed attached to the carbon chain of the small molecules during their condensation. It is not yet possible on the basis of the isotope experiments to establish the mode of reaction with certainty. All schemes proposed for biological fatty acid synthesis postulate aldol condensations and Cannizzaro reactions as intermediate steps. Bonhoeffer and collaborators (8, 9) have shown that these two reactions, when carried out in a medium of heavy water, do not lead to the introduction of stably bound deuterium into the resulting compounds. These findings may explain why such a large proportion of hydrogen in the newly formed fatty acid is normal hydrogen, derived from that of the small molecules which were condensed. The deuterium found in the end-product (fatty acid) may have been introduced in those steps which involved reductions.

#### EXPERIMENTAL

*Feeding Experiments*—Male mice of 20 to 22 gm. weight were used for the experiment. Before injection or feeding of heavy water they were kept on their experimental diet for 24 to 48 hours. They obtained food and water *ad libitum*. All diets were low in fats and high in carbohydrate. The animals of Experiments A, B, and D obtained dried, ground white wheat bread, exhaustively extracted with ether. The animals of Experiment C obtained a mixture of sucrose 76 per cent, agar 15 per cent, extracted meat powder 5 per cent, salt mixture (10) 4 per cent, and vitamins A, D, and B. Only the animals of Experiments A and B, both of which were of very short duration, obtained an injection of 0.5 cc. of concentrated  $D_2O$  prior to the feeding of dilute heavy water, in order to raise the deuterium content of the body fluids immediately to the desired level. The animals of Experiments C and D were not injected, and the deuterium of the body fluids thus rose from zero to the end-value during the experimental period.

*Distillation of Body Fluids and Isolation of Total Fatty Acids*—The mice were killed with ether. Five animals of each experiment were assembled and samples of water were distilled off for deuterium analysis. The values found for each group were practically the same in each experiment, indicating a good reproducibility of the experimental conditions (Table IV).

The livers, intestinal tracts, and kidneys were removed and worked up separately from the rest of the body. The isolation of fatty acids and the separation of saturated and unsaturated fractions were carried out as described before. The results of the deuterium analysis (11) are given in Table I.

*Isolation of Stearic Acid and Palmitic Acid*—5.76 gm. of fatty acids from the saturated fraction of Experiment A were converted into the methyl esters and fractionated in the apparatus described before (6). Of the ten fractions obtained, Fractions 4 and 5, melting at 29.5–30.0°, were combined (1.1 gm.), saponified, and twice crystallized from dilute acetone. Palmitic acid, melting at 61.2–62.0°, was obtained. Fraction 9, 0.66 gm. (m.p. 25–27°), and Fraction 10, 0.22 gm. (m.p. 34°), were combined and separated

TABLE IV

*Deuterium Content of Body Water of Mice Given Heavy Water to Drink*  
Groups of five animals were pooled for each water analysis.

Experiment	Deuterium in drinking water	Duration of experiment	Deuterium in organ water (atom per cent)			
			Group I	Group II	Group III	Group IV
	<i>atom per cent</i>	<i>days</i>				
A	6.75	2	3.68	3.50	3.22	3.46
B	6.75	3	4.21	4.87	4.32	
C	4.0	6	2.53	2.27		
D	4.0	15	2.91	3.00	2.95	2.98

by redistillation into five fractions. Fraction 3, 0.384 gm. (m.p. 36.5–37°), and Fraction 4, 0.102 gm. (m.p. 37°), of this distillation were combined, saponified, and the acid twice recrystallized from dilute acetone. The stearic acid obtained melted at 67–68°. No depression of melting point was observed on admixture with authentic stearic acid.

## SUMMARY

1. The deuterium content of the body water of mice was raised by giving the animals dilute heavy water to drink. The animals were kept on diets low in fat. Saturated and unsaturated fatty acids were isolated from different organs and their deuterium content determined.

2. The deuterium content of the fatty acids of the liver was

always considerably higher than that of the acids of other organs, indicating a rapid regeneration of fatty acids in this organ. Half of the saturated acids are regenerated in about 1 day.

3. In confirmation of earlier results from this laboratory, saturated fatty acids during synthesis in mice acquire a concentration of deuterium half that of the body fluids in which they were formed. The result suggests that during synthesis about 1 out of every 2 stable hydrogen atoms is derived from the water.

4. Stearic acid and palmitic acid were isolated from the depot fat of mice that had been given heavy water for only 2 days. The deuterium contents of the two acids was the same as that of the total saturated acid. The finding indicates that both acids, despite their different abundance in the fat, are regenerated at the same rate.

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## THE CONSTITUTION OF VITAMIN K<sub>2</sub>\*

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The isolation of vitamin K<sub>2</sub> as a yellow crystalline compound melting at 53.5–54.5° has recently been reported from this laboratory (McKee, Binkley, MacCorquodale, Thayer, and Doisy, 1939; McKee, Binkley, Thayer, MacCorquodale, and Doisy, 1939). From the analytical data it was impossible to decide definitely on a formula for vitamin K<sub>2</sub>. The formula C<sub>40</sub>H<sub>54</sub>O<sub>2</sub> was used for all calculations, although C<sub>38</sub>H<sub>50</sub>O<sub>2</sub>, C<sub>39</sub>H<sub>52</sub>O<sub>2</sub>, C<sub>41</sub>H<sub>56</sub>O<sub>2</sub>, and C<sub>42</sub>H<sub>58</sub>O<sub>2</sub> agree with the analytical data equally well. In this paper we shall present evidence to show that the correct empirical formula is C<sub>41</sub>H<sub>56</sub>O<sub>2</sub> and on the basis of degradation studies propose a structural formula.

Upon catalytic hydrogenation the vitamin absorbed 9 moles of hydrogen to produce a colorless compound which on exposure to the air was oxidized to a yellow compound. This yellow compound absorbed 1 mole of hydrogen to give a colorless solution which on oxidation with air returned to the original yellow color. This behavior on hydrogenation as well as its instability toward light and alkali indicated that the vitamin is a quinone. The yellow color of the vitamin is characteristic of 1,4-quinones. Further evidence for the quinone structure of the vitamin was obtained by preparation of the white crystalline diacetate of dihydrovitamin K<sub>2</sub> (Binkley, MacCorquodale, Cheney, Thayer, McKee, and Doisy, 1939). Catalytic hydrogenation of this diacetate caused an uptake of 8 moles of hydrogen. The addition of 12 atoms of bromine by the diacetate of dihydrovitamin K<sub>2</sub>

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indicated the presence of six double bonds in the side chains of the molecule.

The ultraviolet absorption curves (Ewing, Vandenbelt, and Kamm, 1939) for vitamins K<sub>1</sub> and K<sub>2</sub> and for 2,3-dimethyl-1,4-naphthoquinone show a striking similarity; likewise, the curves for the diacetates of the respective hydroquinones show close agreement. This evidence not only supports the conclusion that the vitamin is a 1,4-quinone but, together with the hydrogenation data, indicates that the vitamin is a 1,4-naphthoquinone. Since under the conditions of reduction 3 moles of hydrogen are used to form a tetrahydronaphthohydroquinone there must be six double bonds in the side chains. This is in agreement with bromine addition by the diacetate of dihydrovitamin K<sub>2</sub>.

Vitamin K<sub>2</sub> does not respond to Craven's color test (Craven, 1931). 1,4-Naphthoquinones substituted in the 2 position give a deep blue color with ammoniacal alcoholic ethyl cyanoacetate, while 2,3-disubstituted derivatives give no color change. From this it was concluded that vitamin K<sub>2</sub> was a 2,3-disubstituted 1,4-naphthoquinone with six double bonds in the side chains. Since vitamin K<sub>1</sub> possessed no substituents in the benzenoid ring, it seemed unlikely that vitamin K<sub>2</sub> would be substituted in the benzene ring.

The degradation of the vitamin by chromic acid oxidations as used successfully for the breakdown of vitamin K<sub>1</sub> (MacCorquodale, Cheney, Binkley, Holcomb, McKee, Thayer, and Doisy, 1939) gave only oils from which no crystalline products could be separated. Oxidation of the vitamin with KMnO<sub>4</sub> resulted in the formation of phthalic acid which showed that the benzenoid ring was unsubstituted. However, no intermediate products could be isolated by this method. Several attempts were made to prepare glycols by the addition of hydroxyl groups to the double bonds in the hope that these glycols could then be cleaved by lead tetraacetate. However, dilute KMnO<sub>4</sub>, OsO<sub>4</sub>, and Hg(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub> gave mixtures from which no pure products could be isolated.

In the study of the structure of vitamin K<sub>1</sub> it was found that ozonolysis of the diacetate of dihydrovitamin K<sub>1</sub> in glacial acetic acid followed by decomposition of the ozonide with water gave an excellent yield of 2,6,10-trimethylpentadecanone-14 but the quinone portion of the molecule was attacked so extensively that

it could not be isolated. Subsequently, in a model experiment it was found that the ozonide could be decomposed by the addition of ether and zinc to give an excellent yield of 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde melting at  $115^{\circ}$  (I). It formed a semicarbazone melting at  $206^{\circ}$ .

When vitamin  $K_2$  was treated in glacial acetic acid with ozone and the ozonide decomposed with zinc in ether, a good yield of 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde was obtained in the ether-soluble, water-insoluble fraction. It melted at  $115^{\circ}$  and gave a semicarbazone melting at  $206^{\circ}$ . Mixed melting points showed that this aldehyde was identical with the aldehyde obtained from vitamin  $K_1$ . The isolation of this aldehyde demonstrates conclusively that vitamin  $K_2$  is a 2-methyl-1,4-naphthoquinone.

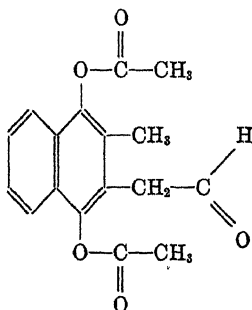
From the water-soluble products of the ozonization reaction levulinaldehyde was isolated as the bis-2,4-dinitrophenylhydrazone (Strain, 1933). Of significance in this case is the yield of levulinaldehyde obtained. On the assumption that 5 moles of levulinaldehyde would originate from 1 mole of vitamin  $K_2$  a yield of 93 per cent was obtained. As a control experiment farnesol ozonized under the same conditions gave a 75 per cent yield of levulinaldehyde.

The third compound isolated from the ozonization reaction was acetone which was identified as the 2,4-dinitrophenylhydrazone and by iodometric titration.

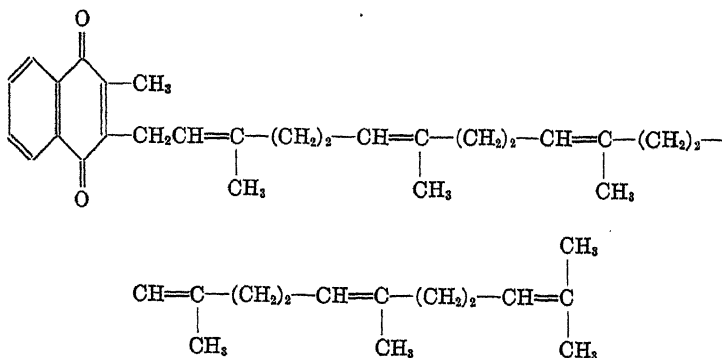
Since the fragments isolated give a total of 41 carbon atoms,  $C_{41}H_{56}O_2$  is proposed as the correct empirical formula of vitamin  $K_2$ . The most probable arrangement of the fragments that make up the side chain in the 3 position is for six isoprene units to be hooked head to tail with a double bond at each carbon atom to which the methyl group is attached, as is the case with farnesol. This side chain may be considered as two farnesyl radicals fastened head to tail. The proposed structure is best expressed by Structural Formula II.

The discovery that vitamin  $K_2$  is also a derivative of 2-methyl-1,4-naphthoquinone is of considerable interest. The most potent antihemorrhagic compound known is 2-methyl-1,4-naphthoquinone. Closely related substances which are also very potent, such as 2-methyl-1,4-naphthohydroquinone and 4-amino-2-

methyl-1-naphthol, are similar in structure and probably are readily convertible in the organism to 2-methyl-1,4-naphthoquinone. Substitution in the 3 position with another methyl radical diminishes the potency a great deal; in fact, 2,3-dimethyl-1,4-naphthoquinone is less active than vitamin K<sub>2</sub> (side chain of 30 carbon atoms in the 3 position), which in turn is exceeded



I. 1,4-Diacetoxy-2-methylnaphthalene-3-acetaldehyde



II. Vitamin K<sub>2</sub> (proposed structure)

in potency by vitamin K<sub>1</sub> which has the phtyl radical (20 C) in the 3 position.

#### EXPERIMENTAL

*Identification of Phthalic Acid*—650 mg. of the diacetate of dihydrovitamin K<sub>2</sub> and 3 gm. of powdered KMnO<sub>4</sub> were dissolved

in 25 cc. of acetone and the solution refluxed for 4 hours. The precipitated  $\text{MnO}_2$  was filtered off and washed with a small amount of acetone. The  $\text{MnO}_2$  was washed thoroughly with 150 cc. of boiling water and the aqueous washing kept separate from the acetone filtrate. The water washings were acidified to Congo red paper and extracted three times with an equal volume of ether. The combined ether extracts were washed twice with a small volume of water and the ether layer evaporated to dryness, leaving 30 mg. of acids. After standing a few hours the product became filled with white needles. The crystals were carefully washed with a few cc. of cold chloroform and then sublimed. The sublimate consisted of long needles, characteristic of phthalic anhydride, melting at  $130\text{--}131^\circ$ . Mixed with an authentic sample of phthalic anhydride melting at  $130\text{--}131^\circ$ , the crystals melted at  $130\text{--}131^\circ$ .

$\text{C}_8\text{H}_4\text{O}_5$ . Calculated, C 64.87, H 2.72; found, C 64.68, H 2.77

*Ozonolysis of Diacetate of Dihydrovitamin K<sub>1</sub>*—200 mg. of the diacetate of dihydrovitamin K<sub>1</sub> were dissolved in 10 cc. of glacial acetic acid and a stream of ozone passed into the solution until no more was absorbed. The solution was diluted with 50 cc. of ether and 0.5 gm. of zinc dust was added in small portions with shaking. After 1 hour the mixture was filtered and the ether filtrate extracted twice with an equal volume of water. The ether was then extracted with sodium bicarbonate solution and finally with water. The ether was then evaporated to dryness, leaving a colorless oil which on cooling became white with the formation of crystals. The product was washed with 4 cc. of cold petroleum ether and the crystalline residue crystallized twice from ether at  $-30^\circ$  to yield 34.7 mg. of white crystals melting at  $115\text{--}115.5^\circ$ .

$\text{C}_{17}\text{H}_{16}\text{O}_5$ . Calculated. C 67.99, H 5.37  
Found. " 67.96, 68.20, " 5.42, 5.51

The material from the mother liquors of the final crystallization (35 mg.) was dissolved in 5 cc. of ethyl alcohol; 35 mg. of semicarbazide hydrochloride and 40 mg. of sodium acetate crystals were added. 3 drops of water were added and the solution warmed in a water bath for 10 minutes and left overnight. On dilution



with water and cooling a gummy precipitate formed. After crystallization first from methyl alcohol and then from ethyl alcohol 21 mg. of a semicarbazone melting at 206–206.5° were obtained.

C <sub>18</sub> H <sub>19</sub> O <sub>5</sub> N <sub>3</sub> .	Calculated.	C 60.49,	H 5.35,	N 11.76
	Found.	" 60.64, 60.49,	" 5.26, 5.46,	" 11.72

*Ozonolysis of Farnesol*—290 mg. of farnesol were ozonized and the ozonide decomposed by the same method as was used for the diacetate of dihydrovitamin K<sub>1</sub>. After the zinc was filtered off, the ether was removed by distillation. The residue was treated with an excess of 2,4-dinitrophenylhydrazine dissolved in 6 N sulfuric acid. After 2 hours the orange precipitate was filtered off, washed with 6 N H<sub>2</sub>SO<sub>4</sub>, and then with methyl alcohol. The dried crude levulinaldehyde bis-2,4-dinitrophenylhydrazone weighed 800 mg., a yield of 75 per cent.<sup>1</sup> After crystallization from a large volume of ethyl alcohol and twice from nitrobenzene it melted at 230–232°.

*Ozonolysis of Vitamin K<sub>2</sub>. Isolation of 1,4-Diacetoxy-2-Methylnaphthalene-3-Acetaldehyde*—866 mg. of the diacetate of dihydrovitamin K<sub>2</sub> were dissolved in 20 cc. of glacial acetic acid and ozone passed into the solution until no more was absorbed. The reaction mixture was diluted with 100 cc. of ether and 4 gm. of zinc dust added in small quantities with shaking over a period of about 1 hour. After the mixture had stood for 4 hours, the zinc was filtered off and the filtrate distilled at 100° to remove the ether and other volatile substances. The residue was diluted with 2 volumes of water and extracted with ether. The ether extract was thoroughly extracted with water, and the water washings combined with the original water layer (volume 235 cc.).

The ether was distilled to dryness to give 433 mg. of an oily product which crystallized on cooling. After two crystallizations from ether the crystals melted at 115°. When the product was mixed with 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde obtained from vitamin K<sub>1</sub>, it melted at 115–115.5°.

C <sub>17</sub> H <sub>15</sub> O <sub>5</sub> .	Calculated.	C 67.99,	H 5.37
	Found.	" 67.82, 67.92,	" 5.42, 5.55

<sup>1</sup> The low yield obtained was probably due to impurities contained in the farnesol.

A semicarbazone melting at 206° was prepared according to the same procedure as was previously employed with the aromatic aldehyde from vitamin K<sub>1</sub>. When mixed with the semicarbazone of 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde obtained from vitamin K<sub>1</sub>, it melted at 206°.

C <sub>18</sub> H <sub>18</sub> O <sub>8</sub> N <sub>3</sub> .	Calculated.	C 60.49,	H 5.35,	N 11.76
	Found.	" 60.39, 60.30,	" 5.35, 5.53,	" 11.84, 11.76

A solution of 80 mg. of the aldehyde in 20 cc. of glacial acetic acid was mixed with 5 cc. of 80 per cent glacial acetic acid containing 20 mg. of CrO<sub>3</sub>. After standing at room temperature for 1 hour the solution was warmed at 60° for 30 minutes. The reaction mixture was diluted with water and extracted with ether. The ether extract was washed with water and then extracted with NaHCO<sub>3</sub> solution. The NaHCO<sub>3</sub> extracts were acidified to Congo red paper and extracted thoroughly with ether. After being washed with water, the ether was evaporated to dryness. The residue was taken up in 10 cc. of methyl alcohol, treated with norit, filtered, and evaporated to 3 cc. 3 cc. of water were added and the solution allowed to crystallize at -5°. Two more crystallizations from 50 per cent methanol gave a white crystalline product melting at 208–209° and when mixed with 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid melting at 209–210°, it melted at 209–210°.

C <sub>18</sub> H <sub>18</sub> O <sub>8</sub> .	Calculated.	C 64.55,	H 5.10
	Found.	" 64.38, 64.40,	" 5.07, 5.17

*Isolation of Levulinaldehyde*—5 cc. of the water extracts (total volume 235 cc.) were added to 30 cc. of 6 N H<sub>2</sub>SO<sub>4</sub> in which 100 mg. of 2,4-dinitrophenylhydrazine had been dissolved and the solution allowed to stand overnight at room temperature. The precipitated hydrazone was filtered off, washed with acetic acid, and then ether. The product was dried in a vacuum desiccator, weight 59.2 mg., m.p. 218–220°. This represents a 93 per cent yield based on the assumption that 1 molecule of the vitamin would give 5 molecules of levulinaldehyde. After one crystallization from nitrobenzene the product melted at 230–232°. Strain (1933) reports a melting point of 235° (corrected) for the bis-2,4-dinitrophenylhydrazone of levulinaldehyde. Mixed with the bis-2,4-dinitrophenylhydrazone obtained from farnesol which melted at 230–232°, the product melted at 230–232°.

C <sub>17</sub> H <sub>16</sub> O <sub>8</sub> N <sub>8</sub> .	Calculated.	C 44.37,	H 3.48,	N 24.33
	Found.	" 44.23, 44.65,	" 3.56, 3.59,	" 24.35

*Isolation of Acetone*—In order to isolate the acetone 726 mg. of the diacetate of dihydrovitamin K<sub>2</sub> were ozonized and the ozonide decomposed with zinc. The zinc was filtered off at  $-5^{\circ}$  and the ether distilled into 25 cc. of 10 per cent NaHSO<sub>3</sub>. The ether layer from the distillate was separated and extracted twice with 25 cc. of 10 per cent NaHSO<sub>3</sub>. The combined NaHSO<sub>3</sub> extracts were treated with 6 gm. of KOH dissolved in 20 cc. of water and the solution distilled, about 60 cc. being collected in the distillate. The distillate was diluted to 100 cc. 10 cc. of this solution were added to 20 cc. of 6 N H<sub>2</sub>SO<sub>4</sub> containing 50 mg. of 2,4-dinitrophenylhydrazine. After standing 4 hours, the yellow precipitate was filtered off and washed with dilute H<sub>2</sub>SO<sub>4</sub> and water. After drying in a vacuum desiccator it weighed 15 mg., m.p. 119–120°. If 1 mole of acetone originates from 1 mole of the vitamin, this represents a 59 per cent yield. After one crystallization from alcohol the derivative melted at 122–123°. When mixed with an authentic specimen of acetone 2,4-dinitrophenylhydrazone melting at 124–125°, it melted at 123–124°.

C <sub>9</sub> H <sub>10</sub> O <sub>4</sub> N <sub>4</sub> .	Calculated.	C 45.38,	H 4.23,	N 23.51
	Found.	" 45.26, 45.30,	" 4.38, 4.33,	" 23.79

Iodometric titration of the solution with 5 cc. aliquots by the method of Hubbard (1920) showed that the total acetone present was 33.3 mg. or a yield of 53 per cent.

A fuchsin aldehyde test on the solution was negative.

#### SUMMARY

Vitamin K<sub>2</sub> was found to be a 2,3-disubstituted 1,4-naphthoquinone with the substituent in the 2 position being a methyl group. Oxidation of the diacetate of dihydrovitamin K<sub>2</sub> with KMnO<sub>4</sub> gave phthalic acid, showing the absence of substituents in the benzenoid ring. Treatment of the diacetate of dihydrovitamin K<sub>2</sub> with ozone and decomposition of the ozonide with zinc in ether gave 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde which was identical with the aldehyde isolated from vitamin K<sub>1</sub> by the same procedure. The yields of acetone and of levulinaldehyde indicated that 1 and 5 moles, respectively,

were produced by the ozonization of 1 mole of diacetyl dihydrovitamin K<sub>2</sub>. C<sub>41</sub>H<sub>56</sub>O<sub>2</sub> is proposed as the correct empirical formula for vitamin K<sub>2</sub> and on the basis of degradation products a structural formula is proposed.

The authors wish to express their appreciation to Dr. C. N. Jordan for the loan of the ozonizing apparatus and to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

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# A RAPID METHOD FOR THE SEPARATION OF SERUM ALBUMIN AND GLOBULIN

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Many attempts have been made to shorten and improve the Howe (1) method for separation of serum globulin from albumin. Howe (2) and Goettsch and Kendall (3) found that errors were caused by the adsorption of albumin by filter paper. These errors were shown by Robinson, Price, and Hogden (4) to vary with the amounts of solution filtered and the kind and size of filter paper used. The loss was greatest in the first portions of the filtrate and it was recommended that these be discarded. Harris (5) evaluated statistically the loss of albumin due to adsorption by filter paper and found it to be 0.05 gm. per cent. To avoid loss of time and errors of filtration, Robinson, Price, and Hogden (6) employed the angle centrifuge to separate the globulin precipitate. They found that globulin precipitated by 1.5 M sodium sulfate solution could be separated quantitatively after standing 4 or more hours by centrifuging at 4200 R.P.M. for 1 hour. These authors (7) also showed that globulin could be salted-out at 25° as well as at 38°.

Because of the limitations of existing methods, especially when large numbers of determinations are made, a further simplification of the procedure for globulin precipitation was made. The modified method is based on the use of ether to decrease the density of globulin precipitated by sodium sulfate. After ether extraction and brief centrifugation, globulin separates in a compact layer at the bottom of the ether phase above the sodium sulfate solution. No preliminary period of standing is required.

## *Method*

Add 0.5 cc. of serum to 7.5 cc. of 23 per cent sodium sulfate in a test-tube (15 cc. capacity and at least 15 mm. diameter),

## 732      Serum Albumin and Globulin Separation

TABLE I

*Determinations of Serum Albumin by New Centrifugation Technique  
Compared with Two Methods of Filtration. Evaluation by  
Biuret and Kjeldahl Methods*

The values, given in per cent, represent single determinations.

Filtrates		Centrifugates by new method		Filtrates obtained by the Harris (5) technique	
Kjeldahl (1)	Biuret (2)	Kjeldahl (3)	Biuret (4)	Kjeldahl (5)	Biuret (6)
5.4	5.1	5.2	5.1	5.2	5.0
4.5	4.7	4.6	4.5	4.9	4.7
4.4	4.6	4.5	4.9	4.8	4.7
4.4	4.4	4.2	4.4	4.1	4.5
4.3	4.5	4.7	4.7	4.7	4.4
4.1	4.1	4.4	4.4	4.3	4.5
3.9	3.8	3.8	4.1	4.0	4.0
3.9	3.5	3.8	3.7	4.0	3.8
3.8	3.7	3.9	3.7	4.0	3.7
3.8	3.7	4.0	3.7	3.7	3.7
3.8	3.7	3.8	3.5	3.7	3.8
3.8*	3.8	3.8	3.8	3.8	3.8
3.7	3.8	4.0	3.8	3.8	3.8
3.6	3.7	3.6	3.7	3.3	3.7
3.6	3.2	3.6	3.5	3.3	3.4
3.5	3.7	4.0	4.1	3.9	4.0
3.3	3.6	3.9	3.3	3.6	3.6
3.2	3.0	3.1	3.2	3.3	3.0
2.9	3.1	3.1	3.3	3.2	3.6
2.5*	2.5	2.7	2.6	2.7	2.6
2.4	2.4	2.4	2.3	2.5	2.3
2.3*	2.3	2.4	2.3	2.4	2.3
2.3	1.9	2.1	2.2	2.3	2.1
1.9	2.3	2.3	2.3	2.5	2.3
1.6*	2.0	1.9	2.0	1.9	2.0
Average... 3.48	3.48	3.59	3.56	3.60	3.57
Standard error of difference between averages of Columns 3 and 5 and 4 and 6.....				±0.246	±0.249

\* Lactescent.

and mix thoroughly by inverting. Add about 3 cc. of ether (U.S.P. quality is satisfactory), and shake vigorously for 20 to 30 seconds. Cap the tube to avoid loss of the ether and centrifuge

5 to 10 minutes at about 2200 R.P.M. After centrifugation, slant the tube so that the tightly packed globulin precipitate floating on the sodium sulfate solution separates from the walls of the tube. Insert a pipette through the ether layer preferably along the lower wall, and withdraw the desired volume of the centrifugate for albumin determination. For analysis by the author's colorimetric biuret method (8), use a 2 cc. sample. If a greater volume is desired, as for example 10 cc. for a macro-Kjeldahl determination, 1 cc. of serum may be used with proportionately increased amounts of reagents in a test-tube 17 to 20 mm. in diameter. Total globulin values are calculated by the difference between serum total protein and albumin. If the sodium sulfate is stored at 37.5°, no difficulty is encountered with crystal formation at room temperatures above 21°.

*Comparison with Other Procedures*—Filtrates of serum were prepared by precipitating globulin at 37.5° from 1 cc. of serum with 15 cc. of 23 per cent sodium sulfate solution and after standing overnight were filtered at 37.5° through one sheet of 11 cm. Schleicher and Schüll No. 602eh paper. Filtrates were also prepared by the technique of Harris (5). Centrifugates were prepared from the same samples of serum by the new method described. The protein of the filtrates and centrifugates was determined both by the macro-Kjeldahl and the biuret methods. The data in Table I show that the albumin values of centrifugates agreed well with those of filtrates prepared by the technique of Harris, while the albumin in filtrates prepared by the Howe technique was 0.09 and 0.12 gm. per cent lower. This loss is in harmony with the 0.05 gm. per cent loss by adsorption on one 7.0 cm. sheet of Whatman No. 50 filter paper reported by Harris. The standard error of the difference between the means of the albumin of centrifugates and of filtrates prepared by the Harris method was not significant. The standard error of measurement ( $s.e.m. = \sqrt{\Sigma d^2/2n}$ ) of the difference of single Kjeldahl and biuret determinations in each group was  $\pm 0.128$  for filtrates,  $\pm 0.107$  for centrifugates, and  $\pm 0.111$  for Harris filtrates (9).

#### DISCUSSION

In a preliminary investigation it was found that a quantitative separation of globulin was effected by mixing 0.5 cc. of serum with



6.5 cc. of 27 per cent sodium sulfate and immediately adding 1 cc. of 15 per cent barium chloride and again mixing. The suspended precipitate of barium sulfate and globulin was separated by centrifugation or by gravity on standing 2 to 4 hours at 37.5°. Usually either procedure gave a clear supernatant solution. However, this method could not be applied to sera generally because a colloidal suspension formed in the presence of an abnormal amount of lipid. Formation of such suspensions could be prevented by extraction of the mixture with ether. It was discovered subsequently that when ether was used barium sulfate was no longer necessary for the rapid separation of the globulin precipitate.

The following experiment offers a possible explanation of the action of ether in accelerating the separation of globulin by sodium sulfate. Globulin was salted-out from 1 cc. of serum by addition of sodium sulfate and after shaking with ether was separated by centrifugation. The precipitate was redissolved in 1 cc. of 3 per cent sodium chloride and evacuated until no odor of ether remained. Centrifugation after addition of sodium sulfate caused no separation of the suspended globulin. However, centrifugation after the addition of ether gave a sharp separation. When a heavier solvent such as carbon tetrachloride was used instead of ether, the globulin separated between the two liquid phases beneath the sodium sulfate solution. Thus the globulin or globulin-lipid complex adsorbed enough of the lipid solvent to change its density sufficiently for separation by centrifugation. However, carbon tetrachloride and many other fat solvents, such as carbon disulfide, xylene, bromobenzene, 1,2,4-trichlorobenzene, dichloroethylene, tetrachloroethane, and benzene, could not be used in the place of ether because the separation of globulin was not complete or a portion of the albumin was precipitated. However, globulin entered and was retained within the above solvents regardless of their density.

Aside from accelerating the separation of globulin, ether did not appear to modify the action of sodium sulfate. Close agreement between centrifugates and Harris filtrates indicated that albumin was not altered. Globulin likewise could not have been altered greatly, since it redissolved in saline as shown above.

No difficulties were encountered in obtaining clear centrifugates. The data presented in Table I represent the albumin concentra-

tions of many types of pathological sera, including several specimens from patients exhibiting hyperlipemia associated with the nephrosis syndrome or with obstruction of the bile ducts. Robinson, Price, and Hogden (6) were unable to obtain clear supernatant solutions from "some pathological sera, such as those from patients with lipoidal nephrosis." Many workers have reported similar difficulties with the Howe (1) method.

In the new method the delay of several hours required for flocculation of globulin by previous methods is dispensed with, and in 5 to 10 minutes of centrifugation a clear solution is provided for albumin determination. If this method is used in connection with the author's quantitative biuret method for protein determination (8), total protein, albumin, and globulin can be determined in about 45 minutes. Further studies on the possible application of the method to spinal fluid and effusions are in progress.

#### SUMMARY

A rapid centrifugal method for the separation of globulin precipitate is presented, based on the use of sodium sulfate in combination with ether. Certain advantages over filtration and previous centrifugal methods are described. The results were found to agree with those of accurate filtration techniques. The new method is superior to previously reported methods for analysis of certain types of pathological sera. The accuracy of the biuret method for the determination of serum proteins has been confirmed.

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# A NEW PROCEDURE FOR QUANTITATIVE ANALYSIS BY ISOTOPE DILUTION, WITH APPLICATION TO THE DETERMINATION OF AMINO ACIDS AND FATTY ACIDS\*

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We shall describe here a quantitative method of analysis of organic compounds in mixtures. The procedure requires the isolation of only a small sample of the substance to be determined, the yield being unimportant. This method is based on the fact that a compound which has an abnormal isotope content is inseparable by the usual laboratory procedures from its normal analogue. If, for example, a deuteropalmitic acid is added to a mixture of isotopically normal fatty acids, and then palmitic acid is isolated, this will be a representative sample of the mixture of the added deuteropalmitic acid and the palmitic acid originally present. From the amount of palmitic acid added ( $x$ ) and its deuterium content ( $C_0$ ),<sup>1</sup> as well as the deuterium content of the isolated palmitic acid ( $C$ ), the amount ( $y$ ) of palmitic acid originally present in the mixture can be calculated from Equation 1.

$$y = \left( \frac{C_0}{C} - 1 \right) x \quad (1)$$

As it is only necessary in this method to obtain a sample large enough for the isotope analysis (5 to 100 mg.), large losses can be afforded in the purification process. This procedure can be used for any substance which can be labeled by an isotopic marker (either a stable isotope or a radioactive one).

The method has already been utilized in this laboratory for

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<sup>1</sup> All isotope concentrations must be given as excess above normal. In this communication we shall deal only with the heavy isotopes of hydrogen and nitrogen, whose normal abundances are 0.02 and 0.37 per cent respectively.

the determination of the leucine content of the proteins of a whole rat (1). Shortly afterwards and independently Ussing (2) also described the general principle of the procedure and determined the leucine content of hemoglobin. He has racemized the hydrolysate and added racemic deuteroleucine. From the deuterium content of the insoluble copper salts he calculated the leucine content to be 29 per cent.<sup>2</sup>

If applied to the determination of optically active substances, this method is complicated by the fact that the optical isomers have in general solubilities different from those of the racemic compounds or mixtures. The synthetic isotopic compounds are always racemic. If, for example, glutamic acid be determined by addition of isotopic *dl*-glutamic acid, only its *l*(+) component will form an inseparable mixture with the normal *l*(+)-glutamic acid in the protein hydrolysate, while the *d*(-)-glutamic acid can be fractionated (as *dl*-) from the *l*(+) form. Therefore in such cases it is necessary to employ one of three procedures: (A) The protein hydrolysate is completely racemized, racemic amino acid is added, and racemic amino acid isolated; (B) the synthetic amino acid is first resolved and only the natural isomer added; (C) racemic amino acid is added and a sample of the natural isomer isolated. We have employed this procedure for the determination of glutamic and aspartic acids, as it is possible in these cases to separate a sample of pure *l* isomer from a partial racemate.

These considerations do not apply to the determination of glycine. It will be necessary to determine which of the three procedures may best be employed for other amino acids.

*Theoretical Errors*—The accuracy of the method depends on three factors: (a) the purity of the compound added, (b) the purity of the compound isolated, and (c) the accuracy of the isotope analysis. The various sources of error will be discussed for the case in which deuterium is employed; analogous considerations apply for  $N^{15}$  or other elements.

<sup>2</sup> The calculations of Ussing seem to be in error. To 3.0 gm. of protein was added 0.392 gm. of *dl*-leucine. Ussing gives his deuterium content not as atom per cent excess, but as excess density of the water formed in the combustion of the compound. The leucine added had an excess density of 513 parts per million and the leucine isolated 225 parts per million. The leucine content on the basis of these values is 17 per cent.

(a) As the weight of the compound added can in general be determined with great accuracy, no error arises from this source. The presence of impurities in the compound added gives rise to an error of variable magnitude, depending on the type of impurity. If the deuteropalmitic acid mentioned above is contaminated with 1 per cent stearic acid of the same deuterium concentration, then this will introduce a 1 per cent error in  $y$ . If this contaminant is normal stearic acid, the error introduced in  $y$  is very small, as  $x$  will be too large and  $C_0$  correspondingly too small. If the impurity is a hydrogen-free substance, then a 1 per cent impurity will cause an error of 1 per cent in  $y$ . In general an impurity of 1 per cent in the added compound gives rise to a final error of 1 per cent or less in the determination.

(b) The effects of impurities in the isolated compound again may be divided into three classes. (1) Contaminants which contain no hydrogen (salts, etc.) introduce no error. (2) The presence of 1 per cent of a normal homologous compound introduces an error of about 1 per cent in  $C$  which, for the case in which  $C_0/C$  is 5, gives rise to an error of 1.25 per cent in  $y$ . The larger  $C_0/C$  is, the smaller the error in  $y$  becomes, approaching 1 per cent as a limit. (3) Contaminants containing the same deuterium excess as the palmitic acid isolated introduce no error in the determination. It is clear that if the conditions are so chosen that all contaminants have approximately the same isotope content as the substance being isolated, the effect of these impurities on the analytical value vanishes.

(c) Above 1 atom per cent excess for deuterium and 0.5 atom per cent<sup>3</sup> excess for  $N^{15}$  the error of a single isotope determination is about 1 per cent. Below these values the error becomes larger. For the best results  $C$  should therefore exceed these values. The ratio  $C_0/C$  should also be as large as possible. In Fig. 1 is plotted

<sup>3</sup> In the mass spectrographic analysis employed for  $N^{15}$  the absolute concentration of the heavy isotope is determined with an error of less than 1 per cent. As the natural abundance is 0.37 per cent, the excess abundance has a larger error. Thus a sample containing 1 per cent of  $N^{15}$  atoms and 99 per cent of  $N^{14}$  has an excess abundance of 0.63 per cent. If the absolute abundance has an error of 1 per cent (0.01 atom per cent  $N^{15}$ ), the excess abundance is in error by 1.5 per cent (0.01 in 0.63). As will be seen in the experimental section of this paper, our  $N^{15}$  determinations seem to have an error of less than 0.003 atom per cent in the range of 0.2 atom per cent excess.

the theoretical error introduced in the analysis by a 1 per cent error in either  $C_0$  or  $C$ . It will be seen that little advantage is gained by increasing  $C_0/C$  above 10. In practice this requires that for optimum results the isotope concentration in the added material be above 5 atom per cent excess.

From the above considerations it may be seen that an error of about 1.5 per cent may be expected for a single analysis under favorable considerations; i.e.,  $C_0 = 5$  per cent or greater,  $C_0/C = 10$ , and the compounds isolated are pure.

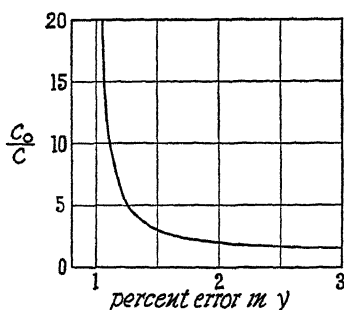


FIG. 1. Error in result caused by a 1 per cent error in either  $C_0$  or  $C$  at different ratios of  $C_0/C$ .

#### EXPERIMENTAL

*Determination of Palmitic Acid in Rat Fat*—The total fatty acids of two rat carcasses were obtained by the usual procedure after hydrolysis of the fats in alcoholic potassium hydroxide for 6 hours. Two aliquots were taken, and deuteropalmitic acid containing 21.5 per cent deuterium was added. The compound was prepared according to the method described earlier (3). The saturated fraction was precipitated as the lead salt from 95 per cent alcohol. The lead salts were twice recrystallized from hot 95 per cent alcohol, decomposed with hydrochloric acid, and the acids extracted with ether. The lead precipitation was repeated and the lead salts again decomposed. The free fatty acids were esterified with methyl alcohol and fractionally distilled. A small sample in the middle of the methyl palmitate fraction was taken for analysis. The ester was saponified, and the free acid recrystallized twice from aqueous acetone. The

constants and deuterium content in the two determinations are given in Table I.

No attempt was made in any step after the addition of deuterio-palmitic acid to obtain a high yield. Relatively large losses were taken in each step to insure purity. The first part of each crystallization was always discarded. While there were present about 3.5 gm. of palmitic acid in each sample, only about 200 mg. were finally isolated and used for analysis.

*Amino Acid Determinations in Protein Hydrolysates*—Estimations of glutamic acid, aspartic acid, and glycine have been made in hydrolysates of cattle fibrin (15.2 per cent nitrogen, without correction for moisture and ash). Approximately 5 gm. samples

TABLE I  
*Determination of Palmitic Acid in Rat Fat*

Total fatty acids	Palmitic acid added*	Properties of palmitic acid isolated				Palmitic acid content calculated
		M. p.		Mol. wt.	Deuterium content	
		Methyl ester	Free acid			
gm.	gm.	°C.	°C.		atom per cent	per cent
14.641	0.2163	29.3-29.5	63.1-63.4	256.4	1.28	23.4
14.135	0.1757	29.0-29.1	63.0	255.4	1.11	22.9

\* The palmitic acid contained 21.5 atom per cent deuterium.

of the protein were hydrolyzed by boiling 20 hours with 150 cc. of 20 per cent hydrochloric acid. To each hydrolysate a weighed amount of optically inactive, isotope-containing amino acid was added. When glutamic acid was added, the hydrolysate plus the added glutamic acid was boiled for 3 hours more to insure that the isotopic glutamic acid and that from the protein would be in the same equilibrium with pyrrolidonecarboxylic acid. After removal of the excess hydrochloric acid, the amino acids were isolated in the usual ways. Glutamic and aspartic acids were precipitated as the barium salts from alcohol. The glutamic acid was separated and purified as the hydrochloride; aspartic acid was precipitated as the copper salt and isolated as the free amino acid. Glycine was isolated as the trioxalatochromiate complex of Bergmann and Fox (4), and purified as the toluene-sulfonyl derivative.



As the added amino acids were synthetic optically inactive compounds, the first crude glutamic and aspartic acids isolated were mixtures of *l*(+) and *dl* components. Specimens of the pure *l*(+) compounds are readily obtained from such mixtures by recrystallization of the glutamic acid as hydrochloride, and of aspartic acid as the free acid.<sup>4</sup> Small samples of the isolated amino acids at successive stages of purification were saved for isotope analysis. The constancy of isotope concentration at the last two or three stages of recrystallization offered strong evidence as to the purity of the compound in addition to that afforded by optical activity and nitrogen content. The data and results of the experiments are given in Table II.

In some experiments Procedure A was tried. The protein was hydrolyzed with barium hydroxide, which yields completely racemized amino acids. In such a case on addition of racemic labeled compounds there would be no question of isolating pure *l* compounds from mixtures of *l* and *dl* components, but simply the preparation of *dl* specimens. The results, however, were unsatisfactory, for with glutamic acid, aspartic acid, and tyrosine there was evidence that these compounds were partially decomposed by boiling with baryta. For if the labeled compound was added before the hydrolysis was begun, the values were always markedly higher than when it was added after the hydrolysis was completed. Further, when the labeled amino acid was present during the hydrolysis, appreciable concentrations of  $N^{15}$  were present in the ammonia given off during the boiling.

Attempts were made to racemize completely the hydrochloric acid hydrolysates with ketene according to the method of Jackson and Cahill (5). In one experiment the isolated glutamic acid was completely racemized and the value 13.7 per cent glutamic acid in the protein was obtained from the isotope dilution, in fair agreement with the results presented in Table II. However, in several other experiments the racemization was only about 90 per cent complete. The data and results of the one successful experiment were as follows: amount of protein hydrolyzed 4.997 gm., *dl*-glutamic acid added 0.4748 gm.,  $N^{15}$  excess in compound

<sup>4</sup> The first crude crop of each substance was recrystallized four or five times in such a manner that the final yield was about 10 to 20 per cent of the crude material.

added 1.957 atom per cent,  $N^{15}$  excess in *dl*-glutamic acid isolated 0.803 atom per cent (the results of five successive recrystalliza-

TABLE II  
*Determination of Amino Acids in Fibrin*

Experiment No.	Protein hydrolyzed	Amino acid added			$N^{15}$ excess in compound isolated at successive stages of recrystallization	Amino acid in protein, found	Evidence for purity of compound isolated
		Compound	Weight	$N^{15}$ excess			
	gm.		gm.	atom per cent	atom per cent	per cent	
17	4.002	Glycine	0.1315	2.00	0.781 0.784 0.782 0.782	5.12	Toluenesulfonyl glycine; found, N 6.09, m.p. 148
21	4.995	<i>dl</i> -Glutamic acid	0.1266	2.10	0.182 0.186 0.185	13.2	<i>l</i> (+)-Glutamic hydrochloride; found, N 7.65, $[\alpha]_D = +25.5$ in 1 N HCl
22	5.382	" "	0.1152	2.10	0.162 0.161 0.157	13.0	<i>l</i> (+)-Glutamic hydrochloride; found, N 7.62, $[\alpha]_D = +25.3$
23*	5.034	" "	0.1222	2.10	0.185 0.176	12.9	<i>l</i> (+)-Glutamic hydrochloride; found, N 7.62, $[\alpha]_D = +25.4$
25†	7.675	" "	0.4005	2.10	0.356 0.355	12.8	<i>l</i> (+)-Glutamic hydrochloride; found, N 7.63, $[\alpha]_D = +25.3$
		<i>dl</i> -Aspartic acid	0.4000	2.00	0.374 0.383	11.2	<i>l</i> (+)-Aspartic acid; found, N 10.44, $[\alpha]_D = +24.9$

\* In this experiment the isotopic glutamic acid was added before the hydrolysis was begun.

† In this experiment both glutamic and aspartic acids were added to the same hydrolysate.

tions were 0.803, 0.800, 0.803, 0.806, and 0.803 atom per cent  $N^{15}$  excess), per cent glutamic acid in the protein (calculated) 13.7.

## DISCUSSION

In agreement with the considerations of the errors to be expected, the determinations of the palmitic acid content of rat fatty acids differ from the mean by 1 per cent. The mean of the four determinations of glutamic acid is  $13.0 \pm 0.1$  per cent. This error (0.8 per cent) is somewhat less than could be expected, as the conditions of the experiments were not those which would give the most accurate results—the concentration of  $N^{15}$  in the starting materials was less than optimal.

The purity of the amino acids isolated is shown not only by the analytical constants, nitrogen content, rotation, and melting point, but also by the constancy of the isotope content of the amino acid in the various stages of purification. This latter criterion is more sensitive to impurities than the nitrogen content or rotation. While 5 per cent of another normal amino acid in the glutamic acid would have only a negligible effect on the nitrogen content, it would change the isotope content by 5 per cent.

The advantages of this method seem to be 3-fold. The error of a determination is independent of the method of isolation or yield, and independent of the concentration of the substance in the starting mixture. No larger percentile error is made whether the absolute concentration of the substance in the mixture is 1 per cent or 50 per cent. Finally the magnitude of the expected error is known; *i.e.*, it is about 1 per cent.

## SUMMARY

A new method (isotope dilution procedure) for the analysis of complex mixtures is described and applied to the determination of glycine, glutamic acid, and aspartic acid in fibrin hydrolysates and of palmitic acid in rat fat.

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## THE GLUTAMIC ACID OF MALIGNANT TUMORS\*

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The claim by Kögl and Erxleben (1) of the occurrence of amino acids of the *d* series, and especially of *d*(-)-glutamic acid, as constituents of cancer proteins has been supported by White and White (2), but refuted by the work of Chibnall *et al.* (3), Graff (4), and Lipmann *et al.* (5). In subsequent papers Kögl, Erxleben, and Akkermann (6) have sought to explain the failures to confirm their work as being due to the inadequacy of the methods used by Chibnall and by Graff for the isolation of glutamic acid. Especially they stress their claim that the Foreman procedure, with either calcium or barium hydroxide, fails to precipitate the *dl*-glutamic acid, alleging that the calcium or barium salt of the *dl*-glutamic acid is much more soluble (10 times) than the corresponding derivative of *l*(+)-glutamic acid.

This paper will offer evidence that such an explanation is not adequate to account for the failures to confirm the work of Kögl and Erxleben. Furthermore, we present the results of a different approach to the problem, namely the use of a modification of the isotope dilution method described in the preceding paper (7), wherein it is clearly shown that, in the six tumors examined, not more than 1 per cent of the glutamic acid could have been in the *d*(-) form.

### *Isotope Dilution Method*

The method for the determination of amino acids in protein hydrolysates (7) has been so modified that the amount of *d*- as

\* This work was aided by grants from the Rockefeller Foundation and the Josiah Macy, Jr., Foundation.

well as of *l*-amino acids can be determined. When *dl*-glutamic acid containing excess  $N^{15}$  is added to a solution of normal glutamic acid, the added isomers will be diluted by their isomeric counterparts in the solution. If the glutamic acid is subsequently isolated by any method and fractionated into two specimens having different optical rotations, *i.e.* containing different proportions of the two optical antipodes, the amount of the dilution of each of the added isomers may be computed from the specific rotation and the  $N^{15}$  content of the specimens isolated. The simplest case is that in which one fraction consists of pure *l*(+)-glutamic acid and the other of the optically compensated *dl* mixture. If  $x$  gm. of racemic compound are added containing  $C_0$  atom per cent excess of  $N^{15}$ , and the *l* and *d* isomers isolated contain  $C_l$  and  $C_d$  atom per cent  $N^{15}$  excess respectively, then the amounts of these isomers originally present in the hydrolysates are

$$y_d = \left( \frac{C_0}{C_d} - 1 \right) \frac{x}{2} \quad (1, a)$$

and

$$y_l = \left( \frac{C_0}{C_l} - 1 \right) \frac{x}{2} \quad (1, b)$$

where  $y_d$  and  $y_l$  are the amounts of *d*(-)- and *l*(+)-glutamic acid respectively in the protein. In the case of glutamic acid it is relatively simple from a partially racemized mixture to isolate the pure *l*- or *d*-glutamic acid, depending upon which isomer is present in excess, and a sample of *dl*-glutamic acid. As in protein hydrolysates the *l* isomer is always in excess, we have isolated *l*- and *dl*-glutamic acid. From the concentration of  $N^{15}$  in the *l*- and *dl*-glutamic acid it is possible to calculate the concentration of  $N^{15}$  in the *d* isomer, for

$$C_d = \frac{1}{2}C_0 + \frac{1}{2}C_l \quad (2)$$

where  $C_d$  is the concentration of  $N^{15}$  in the racemic mixture. Solving for  $C_d$  and substituting in Equation 1, *a*, we obtain

$$y_d = \left( \frac{C_0}{2C_d - C_l} - 1 \right) \frac{x}{2} \quad (3)$$

In two cases we were not successful in isolating a pure *dl*-glutamic acid but obtained glutamic acid having a small excess either of the *l* or of the *d* isomer. From the rotation the percentage of *d* and of *l* component can be determined, and the concentration of  $N^{15}$  in the *d* isomer can be calculated from the equation

$$C_r = PC_l + (1 - P)C_d \quad (4)$$

where  $P$  represents the fraction of the *l* enantiomorph in the sample, and  $C_r$  the concentration of  $N^{15}$  in the incompletely racemized sample. Solving for  $C_d$  and substituting in Equation 1,  $\alpha$ , we find

$$y_d = \left( \frac{C_0(1 - P)}{C_r - PC_l} - 1 \right) \frac{x}{2} \quad (5)$$

In the special case in which there is no *d* component in the protein, the concentration of the isotope in the *dl*-glutamic acid isolated would be half of that in the *l* component isolated plus half of that in the *dl* component added.

#### EXPERIMENTAL

Except in Experiment 31 all specimens were diagnosed as malignant on gross pathological examination and were dissected by the pathologists to exclude non-neoplastic tissue as far as possible. The diagnoses were subsequently verified histologically.

The dried and defatted tissues were hydrolyzed by boiling for 12 hours with 20 parts of 20 per cent hydrochloric acid. In Experiments 34 to 44 inclusive (Table I) 1.20 gm. of *dl*-glutamic acid hydrochloride (1.76 atom per cent excess  $N^{15}$ ) and in Experiment 31 1.00 gm. of *dl*-glutamic acid (1.97 atom per cent excess  $N^{15}$ ) were added. The solution was boiled again for 3 hours to insure equilibration of the added glutamic acid with pyrrolidone-carboxylic acid. Glutamic acid was isolated as the hydrochloride by the Foreman method.

The separation of the *l*(+)- and the *dl*-glutamic acid hydrochlorides from a solution containing the two is based on the fact that the *dl* component is not a racemate but a mixture. The solubility of the *dl* component is just twice that of the *l* component, and a saturated solution of the former is saturated in respect to

TABLE I  
Determination of *l*(+)- and *d*(-)-Glutamic Acid in Tissues

Experi- No.*	Type of tumor or tissue	Tissue taken		Excess N1s in glutamic hydro- chloride isolated				N1s in <i>dl</i> -glu- tamic acid, calcu- lated†		<i>l</i> (+)- Glutamic acid N in total N	<i>d</i> (-)-Glutamic acid in total glutamic acid	Evidence for purity of glutamic hydro- chloride isolated	
		Dry weight	Total N			<i>l</i> form	<i>dl</i> form		per cent			per cent	N
				gm.	gm.		atom per cent	atom per cent		atom per cent	atom per cent		
34	Renal carcinoma (some ne- crosis)	12.0	1.49	0.511	1.137	1.136		7.50	-0.1	<i>l</i> - 7.63 <i>dl</i> - 7.61	+25.3 0		
36	Walker rat carcinoma (little necrosis)	10.6	1.56	0.490	1.120	1.125		7.60	+0.2	<i>l</i> - 7.62 <i>dl</i> - 7.66	+25.3 0		
38†	Secondary ovarian carcinoma (no necrosis)	12.5	1.60	0.517	1.116	1.139		6.88	+1.1	<i>l</i> - 7.62 <i>dl</i> - 7.63	+25.3 0		
40§	Primary adenocarcinoma of fallopian tube (no necrosis)	4.5	0.61	0.911	1.376	1.357		6.98	-1.8	<i>l</i> - 7.64 <i>dl</i> - 7.62	+25.2 -1.25		
42	Flexner rat carcinoma (much necrosis)	9.7	1.06	0.580	1.123	1.139		8.76	+1.0	<i>l</i> - 7.62 <i>dl</i> - 7.65	+25.4 +1.31		
44	Carcinoma of breast, pooled specimens from 10 cases (no necrosis)	10.0	1.40	0.579	1.165	1.169		6.68	+0.2	<i>d</i> - 7.60 <i>dl</i> - 7.63	-25.4 +25.3		
31	Normal term placenta	12.5	1.75	0.542	1.212 1.275	1.256		7.14	+1.8) -0.8)	<i>l</i> - 7.64 <i>dl</i> - 7.61 " 7.62	+25.3 0 0		

\* In Experiments 34 to 44 inclusive 1.20 gm. of *dl*-glutamic acid hydrochloride (= 0.9612 gm. of glutamic acid) having 1.76 atom per cent excess N<sup>15</sup> were added to each hydrolysate. In Experiment 31 1.00 gm. of glutamic acid (1.97 atom per cent excess N<sup>15</sup>) was used.

† This is calculated from Equation 2 or Equation 3 assuming that there was no *d*-glutamic acid in the protein; *i.e.*,  $C_d$  is the same as the concentration of N<sup>15</sup> in the added material.

‡ The primary carcinoma was found in the hepatic flexure of the colon.

§ In this experiment the rotation of the *dl*-glutamic acid hydrochloride indicated about 5 per cent excess of the *d* isomer.

|| In this experiment the rotation of the *dl*-glutamic acid hydrochloride indicated about 5 per cent excess *l*(+) form. In this experiment (as noted in the text) a specimen of pure *d*(-)-glutamic acid hydrochloride was obtained which had excess N<sup>15</sup> 1.73 as compared to 1.76, the excess N<sup>15</sup> in the *dl*-glutamic acid hydrochloride added. This corresponds to 0.8 per cent of the *d*(-) isomer in the total glutamic acid of the protein.



both the *d* and *l* isomers.<sup>1</sup> Such being the case, if the solubility of the *dl* mixture under fixed conditions of temperature and hydrochloric acid concentration is known, then it should be possible to choose the right volume of solvent (aqueous hydrochloric acid) to dissolve the *dl*- but not the excess *l*-glutamic acid hydrochloride. We found the solubility of *dl*-glutamic acid hydrochloride in 5.4 *N* hydrochloric acid at ice bath temperature to be 2.48 gm. per 100 gm. of solution. In practice we used somewhat less than the volume of solvent calculated on the basis of the rotation of the mixture in order to insure complete crystallization of all of the excess *l*-glutamic acid hydrochloride. (Small amounts of *d*-glutamic acid hydrochloride contaminating a specimen of the *l*(+) compound are very readily removed by recrystallization.) In practice the separation did not always go as smoothly as the theory demands, apparently on account of the occasional formation of supersaturated solutions even after shaking for 24 hours in the ice bath. In Experiment 42 the filtrate from the *l*(+) fraction on concentration to small volume yielded glutamic acid hydrochloride containing a considerable excess of the *d*(-) form, enough so that a pure specimen of this form was obtained by recrystallization. The mother liquor from this fraction yielded glutamic acid with a slight excess of the *l*(+) component.

All the preparations brought to analysis for isotope concentration had the correct nitrogen content. The *l*(+) (and the one *d*(-)) specimens had the correct rotation ( $[\alpha]_D = 25.3^\circ$  as the hydrochloride in *N* HCl). The *dl* specimens in not less than 4 per cent solution and in a 2 dm. tube had zero rotation ( $\pm 0.02^\circ$ ) in the last two stages of recrystallization, with the exception of Experiments 40 and 42, in which the rotations indicated about 5 per cent excess *d* and *l* isomers respectively.

#### *Solubilities of Barium and Calcium Glutamates*

*Barium Salts*—0.500 gm. of *l*(+)-glutamic acid hydrochloride and of the *dl*-glutamic acid hydrochloride were separately dis-

<sup>1</sup> This point was brought out by the experiments of Kögl, Erxleben, and Akkermann (6), whose results we have confirmed except that we have found higher values for the solubilities of the *l*(+)- and *dl*-glutamic acid hydrochlorides in both 5.4 *N* and 6.0 *N* hydrochloric acid, either of which might be called 20 per cent. Our values are 1.24 and 0.89 gm. of *l*(+)-glutamic acid hydrochloride per 100 gm. of solution at 0° in respectively 5.4 *N* and 6.0 *N* hydrochloric acid.

solved in 40 cc. of water. The solutions were shaken with excess barium hydroxide and filtered; 5 cc. aliquots of the alkaline filtrate were then pipetted into measured volumes of 95 per cent alcohol, and the flasks stoppered and held in the refrigerator at 0° for 24 hours with occasional shaking. The cold samples were then filtered in the refrigerator and suitable aliquots of the filtrates taken for nitrogen estimation by the Kjeldahl method. At all concentrations of alcohol examined the solubilities of the barium salts of *l*(+)- and *dl*-glutamic acid were identical. The values found were 160, 32.8, 10.3, 6.3, and 4.7 mg. of glutamic acid per 100 cc. of 48, 63, 71, 76, and 80 per cent alcohol respectively. 80 per cent alcohol is the concentration reached when a solution is poured into 5 volumes of 95 per cent alcohol, as is usual in our application of the Foreman procedure. Excess barium chloride, a condition prevailing in the isolation from hydrolysates, reduces solubility by as much as 25 per cent.

*Calcium Salts*—0.3 gm. of glutamic acid hydrochloride in 10 cc. of water was shaken with excess calcium hydroxide (powder). To the mixture, which was alkaline to phenolphthalein, 50 cc. of 95 per cent alcohol were added, and the whole allowed to stand at room temperature for 2 days with occasional shaking. The filtrates were analyzed by the Kjeldahl procedure. Identical experiments were made with *l*(+)- and with *dl*-glutamic acid hydrochlorides. The solubilities found were identical for both forms; namely, 2.2 mg. of glutamic acid per 100 cc. of filtrate.

In a similar experiment in which 2.5 volumes of 95 per cent alcohol were used to precipitate the calcium salts, the solubilities of the calcium salts of *l*- and *dl*-glutamic acid were identical at 4.2 mg. of glutamic acid per 100 cc.

#### DISCUSSION

An extensive consideration of the errors of this method seems unnecessary here. It may be seen from inspection of Equation 2 that, for the values of  $C_{dl}$  and  $C_l$  we obtained, a 1 per cent error in  $C_{dl}$  will give rise to a 1.5 per cent error in  $C_d$ , while the same error in  $C_l$  causes  $C_d$  to be in error by 0.3 per cent. The effect of these errors on the value of  $y_d$  has been discussed in the preceding paper. From these considerations we conclude that the error to be expected in favorable cases is about 1.0 per cent; *i.e.*, a content of *d*(-)-glutamic acid of 1.0 per cent of the total glutamic

acid is within the experimental error. The average of all the tumor experiments is  $-0.1 \pm 0.7$  per cent. Experiment 40 was unfavorable in two respects: (a) the amount of protein hydrolyzed was very small, making an unfavorable ratio of  $C_0/C_i$  or  $C_0/C_d$ , and (b) there was difficulty in preparing a good specimen of *dl*-glutamic acid hydrochloride. Discarding this experiment gives a mean of  $+0.5 \pm 0.5$  per cent. We conclude that in either tumor or normal proteins less than 1 per cent of the glutamic acid is of the *d*(-) form.

#### SUMMARY

By the isotope dilution method, six specimens of malignant tissue were found to contain *l*(+)-glutamic acid in amounts ranging from 6.7 to 8.7 per cent of the total nitrogen. The other isomer (*d*(-)-glutamic acid), if present at all, could not have been more than 1.0 per cent of the total glutamic acid.

Contrary to the statement of Kögl and Erxleben, the solubilities in dilute alcohol of the calcium and barium salts of *dl*-glutamic acid were found to be identical with those of the corresponding salts of *l*(+)-glutamic acid, and therefore cannot account for the failure of Chibnall *et al.* and of Graff to find *dl*-glutamic acid in tumors. Further, it has been demonstrated that the Foreman method will yield *dl*-glutamic acid when it is present.

We are indebted to Dr. A. P. Stout and Dr. C. D. Haagensen of the Department of Surgery, Dr. W. E. Pollard of the Department of Obstetrics and Gynecology, and Dr. M. M. Melicow of the Department of Urology for specimens and for pathological examination.

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## CONCERNING THE METABOLISM OF TESTOSTERONE TO ANDROSTERONE\*

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After the administration of the testis hormone, testosterone, to men with deficient testicular secretion the principal androgen excreted in the urine is androsterone (1, 2). The mechanism of this conversion is obscure with regard to the site of this change, the enzyme systems responsible for the reactions, and the intermediary substances between the testis hormone and the urinary androgen.

Previously, we pointed out that in the conversion of testosterone to androsterone there are six direct intermediates theoretically possible (2). These are etioallocholan-3-one-17-ol, etioallocholan-3( $\alpha$ )-17-diol, etiocholen-(5,6)-3( $\alpha$ )-17-diol, etiocholen-(5,6)-3( $\alpha$ )-ol-17-one, etiocholen-(4,5)-3,17-dione, and etioallocholan-3,17-dione. In this study we have administered three of these possible intermediates, etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, and etiocholen-(4,5)-3,17-dione. In addition to these possible intermediates, we have also attempted to study the metabolism of testosterone, methyltestosterone, androsterone, and dehydroisoandrosterone.

### *Materials, Methods, and Subjects*

The various androgens employed in this study were administered orally in gelatin capsules. A capsule containing one-third of the daily dose was swallowed before each of the three meals.

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All urine samples were collected over complete 24 hour periods, from Case 2 during the 10 days of androgen administration and from Case 4 for 1 additional day thereafter. Between successive periods of ingestion, there was an interval of 10 or more days. Extractions of the urines and the purification of the urinary extracts have been described previously (2). Briefly, the method consisted of extracting the urine with benzene after previous acid hydrolysis. The fraction containing the neutral compounds was separated into ketonic and non-ketonic substances by means of the Girard-Sandulesco ketone reagent (3). The respective fractions containing the ketonic and non-ketonic substances were assayed for androgenic activity and subjected to further chemical purification.

Assays for androgenic activity were performed on the day-old chick (4). All values for androgenic activity are expressed as international units, which is the activity evoked by 0.1 mg. of androsterone.

The subjects were two men with organic and functional evidence of deficient testicular secretion (5), one a eunuchoid and the other a surgical castrate. Case 2, a 26 year-old eunuchoid, has served as a subject in a previous communication (2) and is referred to in this paper by the same designation as used previously. Case 4 is a 44 year-old man who has been bilaterally castrate since the age of 21.

#### EXPERIMENTAL

Table I is a summary of the excretion of ketonic and non-ketonic androgenic substances after the administration of the following compounds: three naturally occurring androgens, testosterone, androsterone, dehydroisoandrosterone; three possible intermediates, etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, etiocholen-(4,5)-3,17-dione; and the orally active androgen, methyltestosterone.

The oral administration of testosterone in the form of its propionate results in increased excretion of androgenic substances in the urine. The greatest increase in androgenic activity was found in the ketonic fraction. For example, in Case 4 when 120 mg. of testosterone propionate per day were administered, the urinary androgens per 24 hours were increased from the pre-

injection level of 7 i.u. to that of 179 i.u. Of the daily average of 179 i.u. of androgenic substance during the treatment period, 160 i.u. were in the ketonic fraction. That at least a portion of this increment is due to androsterone has been previously demonstrated (2).

When methyltestosterone was orally administered in the amount of 105 mg. per day, only a slight increase in androgenic substances was detected in the urine. No change was observed

TABLE I  
*Urinary Excretion of Ketonic and Non-Ketonic Androgenic Substances  
after Oral Administration of Various Androgens*

Case No.	Substance administered	Amount	Androgenic substances in urine, i.u. per day	
			Ketonic	Non-ketonic
		<i>mg. per day</i>		
2	None	0	9	3
4	"	0	7*	
4	Testosterone propionate	120	160†	19
4	Etioallocholan-3( $\alpha$ )-17-diol	105	43	8
2	"	105	103†	63
2	Etioallocholan-3,17-dione	105	87†	7
2	Etiocholan-(4,5)-3,17-dione	105	205†	14
2	Methyltestosterone	105	14	<4
2	"	210	23	<6
2	Androsterone	105	330†	18
2	Dehydroisoandrosterone	105	93	<5

\* Total androgenic activity.

† Androsterone was isolated from these ketonic fractions.

in the non-ketonic fraction; the androgenic activity of the ketonic fraction was elevated from 9 to 14 i.u. per day, amounting to 55 per cent. This relatively small increase is in marked contrast to the excretion of androgenic substance after administration of an equivalent quantity of testosterone, where the increase in ketonic androgens was 1700 per cent. Attention is called to the markedly greater androgenic stimulation, despite low levels of urinary androgens, exhibited upon ingestion of methyltestosterone in comparison to that observed after ingestion of testos-

terone propionate. Evidence of androgenic action was seen in the form of increased erectile ability, skin coloration, and genital growth and weight increase. These changes appeared with each period of ingestion of methyltestosterone, although in lesser degree than upon daily intramuscular injection of 20 mg. of testosterone propionate, and disappeared rapidly when ingestions were discontinued.

When the dose of methyltestosterone was increased to 210 mg. per day, the increase in excretion rose to only 150 per cent. Attempts to isolate the metabolic products of the administered methyltestosterone have thus far been unsuccessful.

The administration of both androsterone and dehydroisoandrosterone in amounts of 105 mg. per day was accompanied by marked augmentation of the urinary androgens. With androsterone the ketonic excretion amounted to 330 i.u. per day, which, if calculated on the basis of androsterone, would amount to 33 mg. With dehydroisoandrosterone the ketonic fraction again accounted for the large increase in androgen excretion, assay of that fraction giving values of 93 i.u. per day.

The ketonic fractions of the urinary concentrates obtained after administration of androsterone and dehydroisoandrosterone were submitted to further purification. Thus far, no metabolic products have been determined on the urine collected after ingestion of dehydroisoandrosterone. After oral administration of androsterone, however, androsterone has been isolated from the urine in good yield.

The complete 10 day output of urine during ingestion of 105 mg. of androsterone daily by Case 2 was hydrolyzed with hydrochloric acid for 15 minutes and extracted quantitatively with benzene. The neutral compounds of the urine extract were separated into ketonic and non-ketonic fractions by means of the Girard-Sandulesco reagent. The viscous brownish red oil which comprised the ketonic fraction was dissolved in hot 80 per cent ethanol, filtered, and finally cooled in an ice-salt mixture. A crystalline precipitate separated, which melted at 168–173°. Sublimation of this material at 125° and 5  $\mu$  pressure yielded a substance which melted at 177–179° and amounted to 222 mg. Two recrystallizations from methanol raised the melting point to 179–181°. The melting point was not depressed by a known sample of androsterone. The identity of the isolated compound

with androsterone was further demonstrated by the preparation of the acetate which melted at 160–163°. When the acetate was mixed with an authentic sample of androsterone acetate, m.p. 161–163°, the mixture melted at 159–162°. From the mother liquors there was isolated a further crop of androsterone amounting to 32 mg., making a total yield of 254 mg. or 24 per cent of the administered substance.

When etioallocholan-3( $\alpha$ )-17-diol was administered by mouth, the greatest portion of the excreted androgens was ketonic in nature. This indicated that etioallocholan-3( $\alpha$ )-17-diol might be converted to androsterone. The fact that the non-ketonic fraction contained considerable androgenic activity in the urine of Case 2 might indicate the presence of considerable unchanged etioallocholan-3( $\alpha$ )-17-diol.

In Case 2 the total output of urine over a 10 day period, during which time 105 mg. of etioallocholan-3( $\alpha$ )-17-diol were ingested daily, was extracted with benzene and the urinary extract separated into the ketonic and non-ketonic fractions. We were unable to isolate any unchanged etioallocholan-3( $\alpha$ )-17-diol from the non-ketonic fraction, but from the ketonic fraction, 33 mg. of androsterone were isolated.

The ketonic substances were separated by means of chromatographic adsorption on an aluminum oxide column with carbon tetrachloride as the solvent. Two fractions were obtained. The first fraction consisted of substances eluted with carbon tetrachloride and the second fraction consisted of compounds eluted with a mixture of 99.7 per cent carbon tetrachloride and 0.3 per cent absolute alcohol. On evaporation of the solvent from the second fraction, sticky yellow-colored crystals were observed. These crystals were recrystallized from 70 per cent ethanol and finally from absolute methanol to yield a white crystalline substance which melted at 179–181°. The melting point was not depressed when mixed with an authentic sample of androsterone. The benzoate of the compound melted at 173–175°; the acetate, at 161–162°.

When etioallocholan-3,17-dione was administered orally to Case 2 in quantities of 105 mg. per day, a total of 94 i.u. of androgenic substances was excreted as compared to the level of 12 i.u. when no androgenic substance was given. By far the greatest quantity of activity was found in the fraction containing the



ketonic substances, 87 i.u. per day as compared to but 7 i.u. for a similar period in the non-ketonic fraction.

The urinary fraction containing the ketonic substances consisted of a viscous oil. This oil was sublimed in a high vacuum and the fraction subliming between 90–135° at 3  $\mu$  was collected. The crystalline sublimate had a melting point of 174–177°. After four recrystallizations from 90 per cent ethanol the melting point was raised to 181–183°. The melting point of a mixture of this substance and an authentic sample of androsterone was 181–183°. The acetate of the isolated compound melted at 163–165°, while the benzoate melted at 174–175°. Neither the melting point of the acetate nor that of the benzoate was depressed when mixed with authentic samples. Thus, the compound is androsterone and a conversion of the administered etioallocholan-3,17-dione into androsterone is indicated.

Oral doses of 105 mg. daily for 10 days of etiocholen-(4,5)-3,17-dione to Case 2 resulted in a great increase in the urinary excretion of androgenic substances. The daily urinary titer of androgens rose from 12 to 219 i.u. The ketonic androgens again accounted for the greatest increases. Upon fractionation of the ketonic substances no unchanged etiocholen-(4,5)-3,17-dione was found; instead androsterone was isolated.

The ketonic substances were sublimed at 120° and 5  $\mu$  pressure. The sublimate was recrystallized from 90 per cent ethanol, and 7 mg. of substance melting at 172–175° were isolated. Water was added to the mother liquor to reduce the alcohol concentration to 80 per cent. A substance separated which was almost colorless and melted at 165–175°. The melting point was raised to 176–178° after two recrystallizations from hexane and the yield was 18 mg. The melting point of the acetate was 162–164°, while the benzoate melted at 174–176°. No depression in melting points was observed when the free compound, the acetate, and the benzoate were mixed, respectively, with authentic samples.

#### DISCUSSION

Each of the seven androgens employed in this study has been shown to be absorbed from the gastrointestinal tract, as evidenced by increased urinary concentration of androgenic substances

over and above that of the pretreatment level. The smallest increase was observed after the administration of methyltestosterone. The percentage absorption of the administered androgens is unknown, since no studies were carried out on the fecal matter.

The urines collected after the administration of five of the seven androgens have yielded crystalline metabolic products. In each case the isolated androgen has been shown to be androsterone. The isolation of androsterone after the administration of testosterone has already been discussed (2). In the case of the ingestion of androsterone, 24 per cent of the administered androgen was isolated unchanged from the urine. This high excretion of unchanged androsterone seems to indicate the relative inability of the body to metabolize this androgen further.

The identification of androsterone in the urine after the administration of etioallocholan-3( $\alpha$ )-17-diol, etiocholen-(4,5)-3,17-dione, and etioallocholan-3,17-dione is of particular interest, since these steroids may be intermediates in the metabolism of testosterone to androsterone. However, the possibility has not been excluded that these compounds may be precursors of testosterone rather than intermediates in the metabolism of testosterone.

#### SUMMARY

1. The excretion of ketonic and non-ketonic androgens in the urine was determined after the oral administration of three naturally occurring androgens, testosterone, androsterone, and dehydroisoandrosterone; of methyltestosterone; and of three possible intermediates in the conversion of testosterone to androsterone. These possible intermediates are etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, and etiocholen-(4,5)-3,17-dione.

2. All seven of the compounds investigated were absorbed from the gastrointestinal tract, as evidenced by increased concentrations of androgens in the urine.

3. By far the greatest increases in androgenic substance were found in the ketonic fractions of the urinary concentrates and appear to be accounted for by androsterone.

4. Androsterone has been isolated from the urine after the oral administration of testosterone, androsterone, etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, and etiocholen-(4,5)-3,17-dione.

We are indebted to Ciba Pharmaceutical Products, Inc., for the supply of androsterone, testosterone propionate (under the trade name perandren), dehydroisoandrosterone, methyltestosterone, etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, and etiocholen-(4,5)-3,17-dione.

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## PREPARATION OF THROMBIN

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Potent preparations of thrombin have been described by Mellanby (1), Howell (2), Bleibtreu (3), and Seegers, Brinkhous, Smith, and Warner (4). The present paper describes a method which, according to our experience, is the most convenient for preparing great amounts of a very potent thrombin.

### EXPERIMENTAL

*Plasma*—Ox blood is obtained at the slaughter-house in glass jars containing 15 cc. of a 20 per cent potassium oxalate solution per liter of blood. As soon as possible after arrival at the laboratory the blood is passed through a metal sieve and centrifuged. There is obtained 50 to 55 per cent (by volume) of plasma, which is used immediately for preparing thrombin.

*Thrombokinese*—Fresh, ground beef lung is extracted with 2 parts of 0.9 per cent NaCl solution for 4 to 5 hours with intermittent stirring. The mixture is then passed through a double layer of gauze and used as such.

*Units*—A thrombin unit is defined as the amount of active substance which will clot 1 cc. of oxalated plasma in 30 seconds at 37°. To 0.10 cc. of the thrombin solution is added 1.0 cc. of ice-cold oxalated plasma and the mixture is placed in a water bath at 37°. For clotting times not greater than 2 minutes the activity is directly proportional to the reciprocal of the clotting time.

*Preparation of Crude Thrombin (A)*—The following procedure was found to give the best results. To 1 liter of oxalated plasma are added 15 liters of cold distilled water and acetic acid until the pH is 5.3 (about 0.3 liter of a 1 per cent solution). After

standing for 5 to 10 minutes the mixture is centrifuged. In the meantime a new lot is precipitated, and this procedure is repeated until in about 1 or 2 hours 10 to 12 liters of plasma have been precipitated and passed through the centrifuge.

The precipitate corresponding to 1 liter of plasma is dissolved at 25–30° in 0.70 liter of a 0.9 per cent NaCl solution. Then 0.15 liter of thrombokinase and 25 cc. of a 1.5 per cent solution of calcium chloride are added (per liter of plasma). The mixture clots in 1 to 3 minutes and is stirred vigorously during this time. After it has stood for 1 hour, the fibrin is removed. The thrombin solution, which contains 6 to 8 thrombin units per cc., *i.e.* about 7000 units per liter of plasma, is then precipitated with 1 volume of acetone and, after standing overnight, is centrifuged. The precipitate is ground in a mortar with acetone and filtered on a Buchner funnel the next day. Again it is ground with acetone and after standing several days filtered and dried with ether. Yield 5.5 to 7 gm. per liter of plasma. About 1200 gm. of crude thrombin were prepared according to this method.

50 mg. of this crude thrombin give in 10 cc. of NaCl solution an activity of 3.5 to 4.0 thrombin units per cc., which corresponds to about 800 units per gm. of substance and 4800 units per liter of plasma; *i.e.*, 70 per cent of the activity of the thrombin solution.

*Purification of Crude Thrombin (B)*—40 gm. of crude thrombin are extracted for 6 hours at 0° with 800 cc. of 0.9 per cent NaCl solution and a few drops of octyl alcohol. After centrifugation the precipitate is extracted again for 12 hours with 600 cc. of NaCl solution. Yield 1100 cc. of a solution containing 30 to 36 thrombin units per cc. 1 per cent acetic acid solution is added until the pH is 5.5. The precipitate is discarded after centrifugation and the solution is poured into 2 volumes of ice-cold acetone. After standing for 2 hours the mixture is centrifuged and the precipitate ground with acetone in a mortar. After further standing for 24 hours it is filtered, treated with absolute alcohol, washed, and dried with ether. Yield 2.5 gm. of purified thrombin containing about 10,000 units per gm., equal to about 80 per cent of the activity in the crude thrombin.

*Properties of Purified Thrombin*—Of the purified thrombin (B) 85 to 90 per cent is soluble in distilled water. Of such a solution

a curve for the precipitation with increasing amounts of ammonium sulfate was obtained according to Schmitz (5) (Fig. 1, Curve I). The curve shows characteristic peaks for globulin and albumin, and, by fractionation with ammonium sulfate two or three times, an albumin fraction could be obtained, which after dialysis contained nearly all the activity; the precipitation curve for this fraction is shown in Fig. 1, Curve II. The globulin frac-

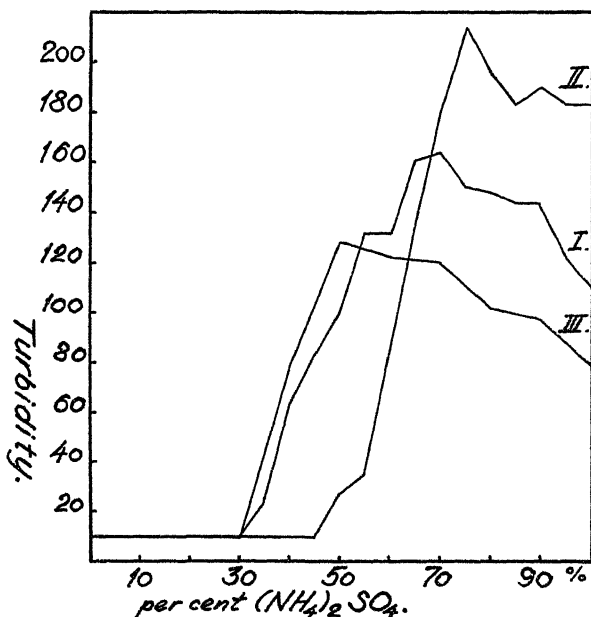


FIG. 1. Precipitation with ammonium sulfate. Curve I, solution of Thrombin B; Curve II, active albumin fraction; Curve III, inactive globulin fraction.

tion was small and practically inactive and gave Curve III, Fig. 1. Precipitating the dialyzed albumin fraction with acetone destroyed about 25 per cent of the activity.

Thrombin thus seems to be connected with proteins of the albumin type. This is interesting in view of the possibility of prothrombin being of globulin character, as it is precipitated by half saturation with ammonium sulfate according to Cekada (6) and Schmitz (7). Work on its purification is in progress.

## SUMMARY

1. A convenient method for preparing great amounts of a potent thrombin preparation is described.

2. Thrombin seems to be an albumin.

Thanks are due to Løvens kemiske Fabrik, Copenhagen, for facilities during this work.

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## LETTERS TO THE EDITORS

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### PARTIAL RACEMIZATION OF GLUTAMIC ACID IN BOILING HYDROCHLORIC ACID SOLUTIONS\*

*Sirs:*

In a recent communication, Johnson<sup>1</sup> has reported the isolation of glutamic acid hydrochloride containing small percentages of *d*(-)-glutamic acid hydrochloride following hydrolysis of Jensen sarcoma and rat livers with 20 per cent hydrochloric acid (30 hours boiling). He suggested that slight racemization of the glutamic acid might have been caused by the hydrolysis. We have tested this hypothesis by boiling *l*(+)-glutamic acid for varying lengths of time in hydrochloric acid solutions. Following the period of heating, the solutions were stored at  $-10^{\circ}$  for 24 to 48 hours, and the glutamic acid hydrochloride crystallizing from them was filtered off, dried, and examined in the polarimeter. In two experiments, a second crop of crystals was obtained by saturating the mother liquors with dry, cold hydrogen chloride. Two of the samples isolated were analyzed in the Organic Micro-analytical Laboratory, University of Minnesota, with the following results.

	C	H	N
Calculated for $C_5H_9O_4NHCl$ . . . . .	32.71	5.49	7.63
Found (for sample with $[\alpha]_D = +6.3^{\circ}$ ) . .	32.72	5.58	7.72
“ ( “ “ “ “ = $+30.3^{\circ}$ ) . .	33.09	5.40	7.75

The data recorded in the accompanying table suggest that the results reported by Johnson can be explained by assuming racemization during hydrolysis. However, some of the results recorded by Kögl and Erxleben<sup>2</sup> are difficult to explain except by assuming the presence of partly racemized glutamic acid residue

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<sup>1</sup> Johnson, J. M., *J. Biol. Chem.*, **132**, 781 (1940).

<sup>2</sup> Kögl, F., and Erxleben, H., *Z. physiol. Chem.*, **258**, 57 (1939).



in the proteins prior to hydrolysis, or by supposing that some property peculiar to the malignant tissue induced an unusually high degree of racemization during hydrolysis (7 hours boiling

Ex- peri- ment No.	Weight of initial glutamic acid	Solvent, HCl 25 cc.	Time of boil- ing	Weight of isolated glutamic acid hydro- chloride	Recov- eries	$[\alpha]_D$	<i>d</i> isomer	Total recov- eries	Total <i>d</i> isomer
	<i>gm.</i>		<i>hrs.</i>	<i>gm.</i>	<i>per cent</i>	<i>degrees</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	9.9572	20%	100	7.8154 1.1426	62.9 9.2	+28.0 +6.3	5.8 40.1	72.1	10.7
2	9.6380	20%	30	10.1802 1.0677	84.6 8.9	+31.4 +15.6	0 25.2	93.5	2.4
3	1.0004	Concen- trated	9	1.0428	83.5	+30.3	2.2	83.5	2.2

with concentrated hydrochloric acid). For example, in one experiment, these authors isolated 6.25 gm. of glutamic acid, containing 42.7 per cent of *d* isomer, from 48.3 gm. of human ovarian carcinoma protein.

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# METHYLATION OF CHONDROSAMINE HYDROCHLORIDE

Sirs:

In course of an investigation on the structure of chondrosin, it was desired to make available the trimethylmethylechondrosaminide. This was prepared by methylation of chondrosamine pentaacetate by the dimethyl sulfate procedure. The N-acetyl-methyltri-O-methylchondrosaminide was then hydrolyzed and tri-O-methylchondrosamine hydrochloride, thus obtained, in its turn was oxidized by means of chloramine-T (Dakin, *Biochem. J.*, **11**, 79 (1917); Herbst, *J. Biol. Chem.*, **119**, 85 (1937)) to 2,3,5-methyl-*d*-lyxose. Thus it follows that on methylation of chondrosamine pentaacetate with dimethyl sulfate, the methylpyranoside is formed.

		C	H	N	COCH <sub>3</sub>
Pentaacetate of chondrosamine.	Calculated.	49.33	5.96	3.59	55.26
	Found.	49.34	6.02	3.62	55.20
N-Acetyltri-O-methylmethylechondrosaminide.	Calculated.	41.92	7.82	5.43	
	Found.	42.02	7.95	5.45	
2,3,5-Trimethyl- <i>d</i> -lyxose.	Calculated.	49.96	8.39		
	Found.	50.13	8.26		
[ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +39.0° (in water at equilibrium) (Bott, Hirst, and Smith, <i>J. Chem. Soc.</i> , 658 (1930))					
[ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +41.7° (in 10 % alcohol + NH <sub>3</sub> )					
2,3,4-Trimethyl- <i>d</i> -lyxose. [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = -22° (at equilibrium) (Bott, Hirst, and Smith, <i>loc. cit.</i> )					

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**Studies on the Optical Activity of Quinine and Its Salts.** BY JAMES C. ANDREWS AND BAILEY D. WEBB. *From the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

For the purpose of providing a micropolarimetric method for the determination of small amounts of quinine, a study has been made of the optical activity of the free base and some of its salts in water, ethyl alcohol, mixtures of the two, and in some other solvents. All samples used were carefully purified, usually by several crystallizations, to effect complete removal of other cinchona derivatives, a constant optical activity under definitely specified conditions being used as the criterion of complete purification. All figures apply at 25°.

The specific activity of the free base in pure ethyl alcohol = -170.0.

The specific activity of the anhydrous sulfate ( $Q_2 \cdot H_2SO_4$ ) = -179.0 in pure ethyl alcohol and -152.5 in pure water. In alcohol-water mixtures the activity rises to a flat maximum of -205.5 in 60 per cent alcohol (by volume).

Similar results were obtained with the dihydrochloride ( $Q \cdot 2HCl$ ). In pure ethyl alcohol its optical activity = -168.8, in pure water -220.5. In alcohol-water mixtures the activity rises to a flat maximum of -228.7 in 30 per cent alcohol (by volume).

Curves showing the effect of progressive neutralization of the free base with HCl and  $H_2SO_4$  have also been run. It is planned to use these figures for maximal optical activity as the basis for a semimicromethod for quinine determination.

**Vitamin K.** BY S. ANSBACHER. *From The Squibb Institute for Medical Research, New Brunswick*

The methods for determining antihemorrhagic activity were analyzed. It was found that 1 Ansbacher unit equals 1 Thayer (1938) unit, 2 Thayer-Doisy (1939) units, 20 Dam units, 0.5 Dann (1938) unit, 1.25 Dann (1939) unit, and 0.16 ml. of Almquist's reference standard.

Since the vitamins  $K_1$  and  $K_2$  have been reported to have a quinoid structure, numerous compounds belonging to the class of quinones were prepared and biologically assayed. It was observed that vitamin  $K_1$  and other phytyl derivatives of methyl-naphthoquinone are not as active and not as readily utilized by the animal body as 2-methyl-1,4-naphthoquinone. This methyl-naphthoquinone is the most potent compound with vitamin K activity and was found to be very rapidly absorbed by the organism. Clinical investigations indicate that it is a highly efficient prophylactic and therapeutic agent for hypoprothrombinemia.

The various postulations concerning the relationship between chemical structure and vitamin K activity will be briefly discussed.

**Individual Phospholipids in Plasma of Rabbits after a Fatty Meal.** BY CAMILLO ARTOM AND JOHN A. FREEMAN. *From the Biochemistry Department, School of Medical Sciences, Wake Forest College, Wake Forest, North Carolina*

By a procedure essentially combining those used by Kirk and by Thannhauser and coworkers, individual phospholipids have been determined in the plasma of rabbits on a standard mixed diet and in the plasma of the same rabbits 15, 30, and 45 hours after the administration of a single large dose of olive oil. In accord with previous results, a clear increase in total phospholipids was only occasionally seen. Moreover, great variability in all the values in oil feeding experiments as well as in controls was apparent. However, higher values for lecithin were very often obtained after oil feeding, and in such experiments as exhibited a clear rise in total phospholipids, the increases were essentially or even entirely in the lecithin fraction. On the contrary, lower values for cephalin were generally encountered in oil-fed animals. The ether-insoluble phospholipids undergo slighter, irregular, and probably meaningless variations.

The above results are believed to point to lecithin as the only phospholipid carrying fatty acids in blood plasma.

**Studies in Iron Metabolism with the Aid of Its Artificial Radioactive Isotope.** BY MARIO E. AUSTONI AND DAVID M. GREENBERG. *From the Division of Biochemistry, University of California Medical School, Berkeley*

With radioactive iron (kindly supplied by Professor E. O. Lawrence and associates) as an indicator, the absorption, distribution, and excretion of this element were studied in normal and iron-deficient rats with hypochromic anemia. The results show that it required about 12 hours for a single dose of iron administered as  $\text{FeSO}_4$  or  $\text{FeCl}_3$  to be removed from the stomach and small intestines.

The passage through the gastrointestinal tract was significantly slower in the anemic rats. The anemic animals absorbed a greater amount of the administered iron and showed a lower excretion in both urine and feces. During a 10 day period the normal animals retained about 30 per cent of the administered iron, while the anemic animals retained 50 per cent. The greater part of the excretion through the feces and the urine takes place within 48 hours.

The absorbed iron was most actively taken up by the bone marrow, blood, and muscle, especially in the anemic rats. After 10 days the radioactive iron nearly disappeared from the muscle and blood of the normal rats but rose to values of 25 and 14 per cent, respectively, of the administered dose in the anemic rats.

**Some Observations on Pantothenic Acid (Chick Antidermatitis Factor).** BY SIDNEY H. BABCOCK, JR., AND THOMAS H. JUKES. *From the College of Agriculture, University of California, Davis*

Pantothenic acid was biologically assayed with chicks on a heated diet. Concentrates of the factor were prepared from extracts of yeast and liver by the methods of R. J. Williams and coworkers. Observations are made on the preparation of such concentrates.

$\beta$ -Alanine and certain hydroxy acids such as lactic acid were subjected to the Schotten-Baumann procedure for coupling amines with organic acids. Some properties of the resulting products are discussed.

The distribution of pantothenic acid in a number of foods for which no data were previously available is reported.

**The Effect of Adrenalectomy on the Deposition in the Liver of Spectroscopically Active Fatty Acids.** BY RICHARD H. BARNES,



ELMER S. MILLER, AND GEORGE O. BURR. *From the Departments of Physiology and Botany, University of Minnesota, Minneapolis*

Spectroscopically active fatty acids which may be accurately differentiated from normal body fats have been utilized for the study of the effect of adrenalectomy on the deposition of liver fat.

It has been shown by others that adrenalectomized rats do not develop fatty livers characteristic of normal rats subjected to certain treatments. A similar effect of adrenalectomy has been demonstrated in rats fed one dose of spectroscopically active fat (tagged fat). 8 hours after the oral administration of approximately 2.0 cc. of tagged fat to adult rats previously fasted for 18 hours there is an increase in the amount of neutral fat present in the liver. Spectroscopic examination of this fat shows that part of this increase in neutral fat is due to the fed fat. In adrenalectomized rats maintained for 4 days with sodium chloride there is no significant rise in neutral fat in the liver. Very little of the neutral fat is derived from tagged fat which has been fed. In neither control nor adrenalectomized animals is there any significant change in the amount of phospholipid present, although a small increase in tagged fat present in this fraction shows that a slow incorporation of tagged fat into the liver phospholipids does take place. Experiments have been conducted to demonstrate the effect of sodium chloride, potassium chloride, and adrenal cortex extract on this deposition of liver fat in adrenalectomized animals.

#### **The Colorimetric Estimation and Fractionation of Androgens in Urine.** BY EMIL J. BAUMANN AND NANNETTE METZGER.

*From the Laboratory Division, Montefiore Hospital, New York*

Previous attempts to apply the Zimmerman reaction for 17-ketosteroids to the estimation of androgens in the urine have been unsatisfactory. The colors obtained from the neutral fraction of benzene extracts of urine with *m*-dinitrobenzene and alkali, particularly from women's urines or those of low androgen content, are quite different from the colors produced by androsterone or dehydroandrosterone. Even the use of color filters, while a great aid in making comparisons, leaves much to be desired.

By purifying the neutral fraction of urine extracts further, partly by solution in pentane in which much gum and pigment

are insoluble, and partly by adsorption on MgO and subsequent elution with ether, we obtained solutions that give colors more like those produced by androsterone or dehydroandrosterone, which are used as standards. Analysis has been extended by dividing these purified extracts into two fractions with digitonin. The insoluble part is probably chiefly dehydroandrosterone.

**The Creatine and Creatinine Excretion of Children during Different Periods of Growth.** BY ELIOT F. BEACH, D. MAXWELL TEAGUE, AND ICIE G. MACY. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

In the course of metabolic studies in this laboratory the daily creatine and creatinine excretions of seven children were determined in 1936 for 10 consecutive days and in 1939 for 55 days. One of the children was also studied in 1932. The age range of the children over all of the periods was from 5 to 12 years and the daily nitrogen intakes varied from 8.5 to 17 gm.

The consecutive daily creatinine values were quite constant. On the other hand, there were wide daily variations in creatine output (from 0 to 130 mg. of creatine nitrogen in one child). This fact demonstrates the advantage in accuracy of an average creatine value for several days over a single 24 hour creatine determination. It was observed that, over the period of growth studied, the total creatine plus creatinine nitrogen output of any one child varied only slightly, while in this relatively constant total, creatine decreased and creatinine increased with increasing age.

The preformed creatinine (nitrogen) coefficients of all the children, at all ages studied, varied from 7.4 to 9.7, which is within the normal adult range. The creatinine coefficient of any individual in the group was almost identical in the two studies.

The creatine output and therefore creatine coefficient decrease in direct accordance with increase in age and are closely related to body surface area, basal metabolic rate, and height. The creatinine output also shows excellent correlation with age, height, and body surface, but unlike creatine is closely related to weight.

**A Comparison of the Effect of Parenteral Injection or Ingestion of Sarcosine versus Glycine, with and without Urea, upon Creatinine-Creatine Excretion in the Rat and Man.** BY

HOWARD H. BEARD. *From the Departments of Biochemistry, University of California, Berkeley, and Louisiana State University School of Medicine, New Orleans*

Sarcosine and urea, alone and together, were injected into rats and ingested by man and the effect of this treatment upon creatine and creatinine excretion in the urine was observed. These results were then compared to those previously obtained under similar experimental conditions when urea and glycine were used.

In the rat the injection of sarcosine and urea alone or together gave from 50 to 71 mg. of extra creatine excretion. Sarcosine injected alone caused an increase of 121 mg. of extra creatinine excretion. In man (H. H. B.) ingestion of urea or sarcosine alone gave no increase in creatinine excretion, while the extra creatine excretion amounted to 13 and 6 gm. respectively. Ingested together the extra creatine excretion was 12 gm. and creatinine excretion 29 gm., or a total of 41 gm. This was 6 gm. more than the amount required by theory and 6 gm. more than that obtained in this subject when similar doses of urea and glycine were ingested. The ingestion of 5 gm. of urea and 5 gm. of glycine by a patient suffering from a very severe muscle atrophy and muscle wasting caused an increase of over 400 per cent in creatine excretion the day after ingestion of these substances. In succeeding days creatine retention occurred.

The above results show that glycine can be first methylated to sarcosine in creatine synthesis in the body. Creatinine can be formed before creatine, and, therefore, may also be an intermediate compound in creatine formation in the body.

**Effect of Cortin, Supracorsin, an Adrenal Hydrolysate, with and without the *d*-Amino Acid Oxidase of Pig Kidney, upon the Appearance, Growth, and Regression of the Walker Sarcoma in Rats.** BY HOWARD H. BEARD. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

160 rats of the Wistar strain were divided into three groups: Group 1, control, 77 animals; Group 2, 68 animals; and Group 3, 15 animals. All were transplanted under nembutal anesthesia with small bits of the Walker sarcoma. For 10 days before and 10 days after transplantation the animals of Group 2 received by

intraperitoneal injection on 1 day 1 cc. of the kidney enzyme preparation, and on the other a mixture of 1 cc. of supracorsin (a water extract of the suprarenal cortex of sheep) + 1 cc. of beef adrenal hydrolysate + 0.5 cc. of Wilson's adrenal cortical hormone. Group 3 received 1 cc. of the kidney enzyme preparation daily.

The results obtained were as follows: (1) 2 weeks after transplantation 58 per cent of the control, 51 per cent of Group 2, and 87 per cent of Group 3 showed the appearance of tumors. (2) Tumors were removed from all groups at the same time and weighed. Forty-four tumors from the control group weighed 451 gm.; twenty-four tumors from Group 2 weighed 188 gm.; and ten tumors from Group 3 weighed 85 gm. The increase in weight of the control tumors was 140 per cent more than that of Group 2. (3) No tumor regressed in the control group. On the other hand there were seven, or 21 per cent, of the tumors of Group 2 which showed complete regression. In addition two other tumors did not grow beyond 1 cm. in diameter. These results were due to supracorsin and the cortical hormone.

**The Synthesis of Glycocyamine and Creatine from Amino Acids, Urea, and Cyanamide.** BY HOWARD H. BEARD AND JULIA K. ESPENAN. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

The *in vitro* synthesis of glycocyamine from urea and glycine and from glycine and cyanamide, and of creatine from sarcosine and urea and sarcosine and cyanamide, with and without the addition of a few drops of strong ammonia, was studied in relation to the effect of time, temperature, and concentration of reacting substances, and the presence of enzymes, upon the process. The speed of the reaction depends, in the order named, upon the temperature, concentration of reacting substances, and time allowed for the reaction to occur. Evidence was obtained indicating the presence of an enzyme in muscle, but not in kidney tissue, catalyzing the above reactions. The synthesis of glycocyamine or creatine, however, does not depend on the presence of the muscle enzyme, provided the ingredients are autoclaved together at 15 pounds pressure for 15 minutes, or the concentration of the ingredients is increased, or a longer time is allowed for the reaction

to occur. The presence of a few drops of strong ammonia greatly catalyzes the synthesis of glycocyamine or creatine under the above conditions. Ammonia should be considered as one of the reacting substances in creatine synthesis from urea and the amino acids in metabolism. Glycocyamine and creatine were isolated and identified as products of the reaction of cyanamide and glycine and cyanamide and sarcosine, respectively. These synthetic substances, when injected into young rats, caused an increase in muscle creatine ranging from 20 to 45 per cent above normal.

**Further Observations upon the Biological Relationship between Creatine and Creatinine in Relation to Water, Salt, and Phosphate Metabolism.** BY HOWARD H. BEARD, JULIA K. ESPENAN, A. LINK KOVEN, AND PHILIP PIZZOLATO. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

Creatinine, in the presence of extra water in the tissues of the rat and man, is hydrolyzed to creatine and any extra creatinine or creatine formed per unit of time is then excreted into the urine. Salt injection or ingestion greatly increases the amount of creatine and creatinine excreted in the rat and man as compared to the same amount of water alone. In the castrate rat creatinine is not hydrolyzed to creatine, but extra creatine is formed from the injection of salt alone. Water and salt cause exactly twice as much extra creatine excretion in the rat as the injection of either alone. Tissue creatine is not increased under these conditions. Alkalosis greatly increases the creatinine  $\rightarrow$  creatine transformation and excretion in the rat. The effect of the sex hormones and alkalosis upon the creatinuria was probably due to water and salt retention. The creatinine  $\rightarrow$  creatine transformation *in vitro* (37 mg.) and *in vivo* (36 mg.) showed excellent agreement. The synthesis of creatine in the body in relation to phosphate and muscle metabolism was discussed and any condition, or set of conditions, that influences the general body metabolism will, at the same time, influence creatinine-creatine equilibrium.

**Creatine-Creatinine Metabolism and the Hormones. III. Effect of Parenteral Injection of Creatine and Creatinine with the Sex**

**Hormones upon Creatine-Creatinine Excretion in Normal Animals.** BY HOWARD H. BEARD AND ERNEST J. JACOB.  
*From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

Different amounts of creatine and creatinine, with and without testosterone propionate and theelol, were injected into normal rats and the effect of this treatment upon creatine-creatinine excretion was observed. The results obtained confirmed our previous findings, as follows: creatine is not changed into creatinine in the rat; the sex hormones injected with creatine likewise do not increase the creatinine excretion; the injection of creatinine stimulates the excretion of creatine and this function of creatinine is increased when it is injected with the sex hormones; no difference in the effect of the sex hormones upon creatine-creatinine metabolism in rats has been observed.

**Creatine-Creatinine Metabolism and the Hormones. IV. Effect of Parenteral Injection of the Sex Hormones, with and without Creatinine, upon Creatine-Creatinine Excretion in the Ninety Day Castrate Rat.** BY HOWARD H. BEARD AND ERNEST J. JACOB. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

The sex hormones, testosterone propionate and theelol, with and without creatinine and saline, were injected into rats 90 days after castration and the effect of this treatment upon the excretion of creatine in the urine was studied. No effect was observed under these conditions upon the excretion of creatine which is in contrast to the creatine excreted in the normal animal or recent castrate under similar experimental conditions. The creatinuria observed was due to the effect of saline alone. It was suggested that secondary changes in some of the other endocrine glands may account for these findings. A summary of results obtained when different hormones are injected in normal and castrate animals upon creatine-creatinine metabolism is given.

**The Effect of Titanium on Sulfur Metabolism.** BY MARY L. C. BERNHEIM. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

Since the oxidation of  $-SH$  groups to  $-SO_3H$  in rat liver in

*vitro* is inhibited by traces of Ti,\* the effect of Ti on the ratio, total  $\text{SO}_4$  to total S, in rat urine was determined. A suspension of  $\text{Ti}(\text{OH})_4$  (1.5 mg. per kilo) was injected intraperitoneally. During the following 24 hours the excretions of total N, total S, and  $\text{SO}_4$  were all less than normal, but the  $\text{SO}_4$  excretion was relatively much lower, giving a very low ratio of total  $\text{SO}_4$  to total S. This effect was compared with that obtained by fasting the rat for 24 hours. The total N and total S excretion was reduced even more than after Ti, but the ratio of total  $\text{SO}_4$  to total S was only very slightly lowered. The following are typical figures: total N (mg. per 24 hours) normal 204, fasted 100, after Ti, 125; ratio of total  $\text{SO}_4$  to total S, normal 0.67, fasted 0.52, after Ti, 0.26. A rapid return to normal of the N excretion after the Ti injection showed that the liver was not permanently damaged.

The low ratios obtained after treatment with Ti cannot be the result of the failure of the rat to eat, but are probably caused by the inhibition by the metal of the oxidative enzymes concerned with sulfur metabolism.

**Structure of Vitamin  $\text{K}_2$ .** BY S. B. BINKLEY, R. W. MCKEE, SIDNEY A. THAYER, AND EDWARD A. DOISY. *From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

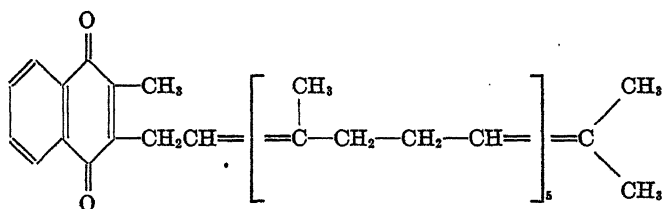
Evidence has been previously presented to show that vitamin  $\text{K}_2$  is a 2,3-disubstituted naphthoquinone with six double bonds in the side chains. Since the vitamin does not react with maleic anhydride, the presence of conjugated double bonds is not likely. A model experiment in which the diacetate of dihydro vitamin  $\text{K}_1$  was ozonized in glacial acetic acid and decomposed by zinc in ether gave an excellent yield of 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde which was further characterized as the semicarbazone. Ozonization of the diacetate of dihydro vitamin  $\text{K}_2$  under the same conditions gave 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde. Analysis and mixed melting point showed this aldehyde as well as its semicarbazone to be identical with the same compound obtained from vitamin  $\text{K}_1$ . The isolation of this aldehyde shows conclusively that vitamin  $\text{K}_2$  is a 2-methyl-1,4-naphthoquinone.

\* Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **127**, 695 (1939).

From the ozonization reaction levulinaldehyde was isolated as the bis-2,4-dinitrophenylhydrazone in 81 per cent yield, based on the assumption that 1 mole of the vitamin would yield 5 moles of levulinaldehyde.

Acetone was also isolated from the ozonization mixture in 56 per cent yield, assuming that 1 mole of acetone originates from 1 mole of vitamin K<sub>2</sub>. The acetone was characterized as the 2,4-dinitrophenylhydrazone and by iodometric titration.

On the basis of analyses, molecular weight determinations, and degradation products C<sub>41</sub>H<sub>56</sub>O<sub>2</sub> is proposed as the correct empirical formula for vitamin K<sub>2</sub>. The proposed structure is best expressed by the following formula.



**Influence of Induced Hibernation on the Growth Behavior of Mouse Neoplasms.** BY FRITZ BISCHOFF, M. LOUISA LONG, AND J. JEROME RUPP. *From the Chemical Laboratory, Santa Barbara Cottage Hospital Research Institute, Santa Barbara*

It has been found possible by subjecting mice to a sudden low environmental temperature,  $-2^{\circ}$  to  $-5^{\circ}$ , to reduce the body temperature below  $20^{\circ}$  for 7 hour periods on 5 successive days or for a continuous 24 hour period, whereby a condition resembling hibernation was produced. In the range of skin temperature (inguinal),  $15-20^{\circ}$ , the animals lose consciousness, respiration is considerably lessened and erratic, and the heart-beat is reduced to about one-half the normal beat. The blood sugar falls below normal in the initial stage. It requires about 2 hours to arrive at this stage. By lowering the environmental temperature  $10-15^{\circ}$  below room temperature ( $20^{\circ}$ ) for 10 to 15 day periods no hibernation resulted. The growth behavior of Sarcoma 180 and the spontaneous Marsh-Buffalo adenocarcinoma has been studied in thirty-two mice subjected to hibernation. Matched tumors were used as controls. No permanent effect upon the growth process



was noted. A temporary growth retardation, of the same order as that produced by caloric restriction, was indicated. Loss of body weight during the hibernation period was about 15 per cent.

**The Microestimation of Alanine and Threonine.** BY RICHARD J. BLOCK, DIANA BOLLING, AND MERRILL WEBB. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, New York*

10 mg. of protein are hydrolyzed with 8 N  $\text{H}_2\text{SO}_4$ ; the  $\text{H}_2\text{SO}_4$  is removed by an excess of barium acetate. After removal of the  $\text{BaSO}_4$  the combined filtrates and washings are concentrated to dryness and dissolved in 50 per cent acetic acid. An aliquot containing 1 or 2 mg. equivalents of protein is oxidized with lead tetraacetate or sodium periodate (pH 8) at  $30^\circ$ . Under these conditions threonine alone is converted quantitatively to acetaldehyde. The latter is aerated into concentrated  $\text{H}_2\text{SO}_4$  containing an excess of *p*-hydroxydiphenyl. The condensation of acetaldehyde with *p*-hydroxydiphenyl yields a permanent red-violet color with an absorption maximum at 560  $\mu$ .

The estimation of alanine is carried out in the following way. An aliquot of the protein hydrolysate equivalent to 0.5 or 1.0 mg. of protein is deaminated with  $\text{NaNO}_2$  and the excess  $\text{HNO}_2$  is removed by warming. The solution is oxidized with lead tetraacetate as described above and the theoretical quantities of acetaldehyde are formed from alanine and from threonine. Alanine is then estimated by the difference in the amounts of aldehyde produced before and after deamination.

**Influence of Parathyroid Deficiency on Reproductive Success in the Albino Rat.** BY MEYER BODANSKY AND VIRGINIA B. DUFF. *From the John Sealy Memorial Research Laboratory and the Department of Pathological Chemistry, School of Medicine, University of Texas, Galveston*

Parathyroidectomized rats, maintained on a diet which assures maximum reproductive success in normal rats (Diet 7 of Cox and Imboden), showed a marked reduction in the per cent of fertile matings, a moderate reduction in the number of young per litter, and a very conspicuous reduction in the average size of the rat pups at term. The combination of parathyroid deficiency and a diet low in calcium (Diet 26 of Cox and Imboden), or high in

phosphorus (Diet 10 of Cox and Imboden), resulted in extremely low grades of fertile matings (less than 5 per cent). Parathyroid deficiency interferes with spontaneous delivery and is associated with a high rate of maternal mortality. The severe manifestations of late pregnancy appear to be related to hypocalcemia and may be averted by altering the mineral composition of the diet.

**Nutritive Value of Zein Hydrolysates.** BY RAYMOND BORCHERS AND CLARENCE P. BERG. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Sulfuric acid hydrolysates of zein were prepared by refluxing the protein with 25 per cent acid (by volume) for 16 hours (I) or heating it in the autoclave with 14 per cent acid at 140° for 7 hours (II), with 10 per cent acid at 165° for 8 hours (III), or with 14 per cent acid at 180° for 15 hours (IV). In Hydrolysates I and II the percentage of amino nitrogen based on total nitrogen was about the same (71 and 69); in Hydrolysate III the percentage was lower (about 60) and in Hydrolysate IV still lower (about 55), indicating possible amino acid destruction or polymerization.

Such hydrolysates, freed of sulfuric acid, were supplemented with lysine, tryptophane, histidine, and cystine and fed to rats as the protein in a diet otherwise adequate. Hydrolysates I and II allowed slow growth equal to that on whole zein, but Hydrolysates III and IV did not support growth. Additions of threonine to the diets containing whole zein or Hydrolysate I or II did not appreciably accelerate the growth rate; similar addition to the diet containing Hydrolysate III induced slow growth, but to the diet containing Hydrolysate IV, little or no change.

Analyses by the method of Block and Bolling indicate that zein contains threonine in an amount which is critically low for growth and that the conditions used in preparing Hydrolysates III and IV destroy most, if not all, of the threonine. In Hydrolysate IV significant amounts of other essential amino acids may also have been destroyed.

**Glycocyamine and Methionine As Precursors of Creatine in Animal Tissues.** BY HENRY BORSOOK AND JACOB W. DUBNOFF. *From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*  
The formation of creatine *in vitro* in the liver and kidney of

seven animals—cat, dog, frog, guinea pig, pigeon, rabbit, and rat—was studied. The tissue slice technique was used; true creatine was distinguished from other Jaffe reagent chromogens by means of the bacterial enzyme suspension of Miller and Dubos.

The livers of all the animals converted glycocyamine to creatine. The increases with glycocyamine were from 5 to 30 times the spontaneous increases or the blank values. This, apparently, is a general property of liver, and suggests that methylation of glycocyamine in the liver is one of the normal sources of creatine. The kidneys of the guinea pig and pigeon also methylated glycocyamine, but at a slower rate than in the liver. The kidneys of the other five animals were negative in this respect.

Creatine formation in the liver from glycocyamine was increased by methionine in all the animals except the guinea pig and rabbit. No such effect of methionine was observed in the kidney of any of the animals except the pigeon.

Slight creatine formation from preexisting precursors was observed in the liver of the guinea pig, pigeon, and rat, and in the kidney of all animals except the pigeon. The absolute increases over the blanks were small.

Liver and kidney tissue (rat) ground so that all cells were disintegrated produced no creatine (or dubious small increases) from preexisting precursors, and none from glycocyamine, with or without added methionine.

**Canine Cystinuria. VI.** BY ERWIN BRAND AND GEORGE F. CAHILL. *From the Departments of Biochemistry and of Urology, College of Physicians and Surgeons, Columbia University, New York*

Further progress has been made in the attempt\* to establish a cystinuric strain† of Irish terriers. Cystinuria in these dogs is not present at birth, but appears around maturity. Information concerning the development of the disorder is not yet available. The data are not sufficient to allow a genetic interpretation of the mode of inheritance.

Litter 4 consisted\* of two cystinuric males and three normal

\* Brand, E., Cahill, G. F., and Slanetz, C. A., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. xvi (1938).

† Brand, E., Cahill, G. F., and Kassell, B., *J. Biol. Chem.*, in press.

females. Mating of a cystinuric male from this litter with its normal sisters yielded four litters. In two of these litters from one female no cystinuric animals were found at maturity (several dogs showed a cystine excretion slightly higher than normal). In a litter from the other female two males and one female developed cystinuria at about 11 months of age (cystine sulfur accounted for 11 to 23 per cent of the total urinary sulfur). A second litter from this female has not yet reached maturity.

Back-crossing of several females from these litters with their cystinuric sire has yielded five litters which are still under investigation.

It is hoped that during the next few years a larger number of cystinuric dogs will be obtained, which will be made available to other research workers.

As a by-product, a considerable number of normal, highly inbred dogs have been produced. With these dogs a "standard" breed for scientific purposes may be developed; some of the "standard" dogs are already being used by other laboratories.

### **The Effect of Thiamine on Pancreatic Lipase and Spinal Cord.**

BY NATHANIEL BROWER AND SHIRO TASHIRO. *From the Biochemistry Department, University of Cincinnati, Cincinnati*

Following the report\* of the presence of a demyelinating agent in the spinal fluid and urine accompanying multiple sclerosis, an attempt was made to find whether such spinal fluid manifested a greater lipolytic activity. 1 cc. of such spinal fluid showed no appreciable activity but it enhanced the hydrolysis of olive oil by steapsin. This means either that the spinal fluid of this disease has more lipolytic accelerator, or has less antilipolytic substance.

Sasaki† found that a decrease of phospholipid content of nervous tissue of vitamin B<sub>1</sub>-deficient albino rats could be restored appreciably by treatment with vitamin B<sub>1</sub>, and, since thiamine is used in the treatment of multiple sclerosis, we investigated to determine whether thiamine acts as an antilipolytic agent. The results show that hydrolysis of olive oil by pancreatic lipase is diminished approximately to 80 per cent with 9  $\gamma$  per 15 cc. and 50 per cent

\* Weil, A., Luhan, J. A., and Balser, B. H., *Tr. Am. Neurol. Assn.*, 142-144 (1935).

† Sasaki, Y., Thesis, University of Cincinnati (1936).

with 300  $\gamma$  per 15 cc. This inhibitory action of thiamine against the demyelination of spinal cord by steapsin will be demonstrated.

**The Stabilization and Determination of Blood Pyruvate.** BY ERNEST BUEDING AND HERMAN WORTIS. *From the Psychiatric Division, Bellevue Hospital, New York*

A significant decrease in pyruvic acid was found after 1 minute if blood was allowed to stand at room temperature before precipitation with trichloroacetic acid. Since the precipitation of blood following its withdrawal requires about 30 to 90 seconds, the necessity of using some stabilizing agent prior to precipitation is obvious. Sodium iodoacetate in a concentration of 0.2 per cent was found to inhibit completely the disappearance of pyruvate *in vitro* and was therefore used as a stabilizing medium for the determination of blood pyruvate. The method of Lu\* was then modified in the following ways: (1) Larger amounts of blood were used. (2) The dinitrophenylhydrazine of the pyruvic acid, together with the free hydrazine, was extracted first with 4 ml. and then twice with 2 ml. of ethyl acetate. (3) 2 N sodium hydroxide was added to the sodium carbonate extract. The blood pyruvate level in 60 normal subjects varied from 0.77 to 1.16 mg. per cent (average 0.98 mg. per cent). Sodium cyanide increased the disappearance of pyruvic acid *in vitro* and this disappearance was not prevented by sodium iodoacetate. Sodium fluoride had neither an inhibiting nor an accelerating effect on the disappearance of pyruvic acid *in vitro*. The pyruvic acid content of the cerebrospinal fluid remained constant even if samples were allowed to stand at room temperature for 1 hour. The correlation of these findings to the functioning of cocarboxylase is discussed.

**Acylation of Amino Acids in Pyridine.** BY HERBERT E. CARTER, PHILIP HANDLER, AND CARL M. STEVENS. *From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana*

The acylation of amino acids in pyridine has been attempted by several workers, but with little success. Since the use of pyridine as an acylating medium has certain advantages, particularly in the

\* Lu, G. D., *Biochem. J.*, **33**, 249 (1939).

case of acyl chlorides of low molecular weight, we undertook a study of the reactions of amino acids with acid chlorides in pyridine. It was discovered that this method of acylation is limited by the insolubility of certain amino acids (tyrosine, serine, threonine, and cystine) in pyridine, and by certain complicating side reactions, the extent of which, to a large degree, depends on the nature of the acid chloride used. Valine, phenylalanine, and leucine, when suspended in pyridine at room temperature, react readily with acid chlorides. The yield of acylated amino acid obtained, with use of equivalent quantities of amino acid and acid chloride, varied from 40 to 75 per cent in the case of aliphatic acid chlorides. The yield was not increased by varying the ratio of acid chloride to amino acid. In the case of benzoyl chloride the yield of benzoyl derivative was very low. The main product consisted of a benzoylated dipeptide. A study of the mechanism of production of the dipeptide leads to the discovery of the rather complex series of reactions which may take place when an amino acid and an acid chloride are allowed to react in the presence of pyridine. These side reactions occur in varying amounts with different acid chlorides. In the majority of cases, however, they are of sufficient importance to render the use of pyridine as an acylating medium rather unsatisfactory.

**The State of Calcium and Magnesium in Blood Serum.** BY  
ALFRED CHANUTIN, STEPHAN LUDEWIG, AND A. V. MASKET.  
*From the Biochemical Laboratory, University of Virginia,  
University*

Serum was centrifuged for 7 hours in an ultracentrifuge capable of exerting a force of about 90,000 times gravity. The serum was divided into four or five fractions and the calcium or magnesium, total protein, albumin, and globulin, and total solids were determined in the respective samples.

The relationship of calcium and magnesium and protein was determined. It was possible to estimate the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentrations in a large number of serums obtained from patients. The values for calcium ion concentrations and for  $\text{pK}_{\text{CaProt}}$  obtained by physicochemical means are similar to the values reported by McLean and Hastings. The combining power of albumin and globulin for calcium was studied.

**A Microscopic Study of the Effects of Choline Deficiency in Young Rats.** BY KERMIT CHRISTENSEN. *From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

Recent studies\* on choline deficiency in young rats have shown that in addition to the production of a fatty liver, a hemorrhagic degeneration of the kidneys occurs, also involution of the thymus gland, enlargement of the spleen, transformation of lymph nodes to hemolymph nodes, and in the more severe cases, hemorrhage in the eye. The chief effect in the liver is the storage of fat by the liver cells. The renal hemorrhage is localized in the capsule and in the connective tissue between the tubules in the peripheral parts of the cortex. Cortical blood vessels beyond the glomeruli were involved and glomeruli are not affected unless in a particularly hemorrhagic zone. Necrosis of the kidney tubules is found in the peripheral parts of the cortex and more deeply the tubules show degenerative changes. At the boundary of the cortex and medulla, the tubules contain hyalin casts and, in the most severely affected animals, casts are present in the papillary ducts. The changes in the thymus gland resemble normal involution. In the smallest thymus glands no cortical tissue is left. The enlarged spleen shows evidence of much congestion, particularly in the red pulp. The appearance of blood in the sinuses of the lymph node is suggestive of a change to a hemolymph node-like structure. Hemorrhage in the eyes occurs mainly from blood vessels in the ciliary body and in the iris.

**Transamination in Liver and Kidney.** BY PHILIP P. COHEN. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The reaction between  $\alpha$ -ketoglutaric acid and different amino acids to form glutamic acid (transamination) has been studied in liver (rabbit) and kidney cortex (pig). The importance of these two tissues in amino acid metabolism makes such a study particularly desirable. Glutamic acid was determined by the author's method.

The percentage of added  $\alpha$ -ketoglutaric acid converted into glutamic acid in the presence of various amino acids for kidney

\* Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939).

and liver respectively is as follows: (1 part of minced tissue + 3 parts of saline-phosphate; 3 ml. of tissue in each cup; gas phase,  $N_2$ ; incubation time 60 minutes; temperature  $38^\circ$ ; substrate concentration, 0.02 M (0.004 M for *dl*-amino acids)) *l*(-)-aspartic acid, 55, 46; *l*(+)-alanine, 35, 42; *l*(+)-arginine, 19, 8; *l*(+)-isoleucine, 18, 4; *l*(-)-leucine, 10, 8; *l*(+)-valine, 15, 12; *l*(-)-phenylalanine, 8, 8; *l*(-)-cysteine, 2.8, 0; glycine, 0, 0; *dl*-methionine, 15, 0; *l*(-)-proline, 0, 0; *l*(-)-tryptophane, 0, 0; *l*(+)-lysine, 3.6, 0; *dl*-histidine, 0, 6.6; *d*(+)-phenylalanine, 0, 0; *d*(-)-isoleucine, 0, 0; *d*(+)-leucine, 0, 0.

It appears from these data that more amino acids react with  $\alpha$ -ketoglutaric acid to form glutamic acid in liver and kidney than in pigeon breast muscle.\* As in pigeon breast muscle, the reaction is most active with *l*(-)-aspartic acid and *l*(+)-alanine. None of the amino acids of the *d* series has been found to be active. The nature of the enzyme systems involved in transamination is at present under investigation.

**The Effects of Strontium and Calcium on the Uterus.** BY VERA V. COLE AND BEN K. HARNED. *From the Laboratory of Pharmacology, Woman's Medical College of Pennsylvania, Philadelphia*

A comparison of the effects of strontium and calcium on the muscular activity of isolated uterine strips from rats, guinea pigs, and dogs has demonstrated that in the presence of appropriate ratios of the two ions the number of maximal contractions is approximately double that in an equimolar concentration of calcium alone.

Uteri from non-pregnant and pregnant animals were used and the cyclic condition of the uterus or the progress of the gestation was noted. Except for the dogs, death was by concussion. Corresponding strips of the uterus were placed in identical portions of oxygenated Locke's solution,  $37.5^\circ$ , containing in separate experiments varying amounts of calcium. The activity of the two sections was recorded simultaneously, and after an appropriate control period, during which the strips duplicated each other satisfactorily, strontium was added to one bath and calcium to the other. A striking difference between the ions was obtained with

\* Cohen, P. P., *Biochem. J.*, **33**, 1478 (1939).



the rats, guinea pigs, and dogs, but there was only a questionable distinction with rabbits. The effects were not due to barium contamination. The chlorides, acetates, and nitrates were employed, and the pH of the uterine bath was controlled by passing the oxygen through 1.5 per cent sodium bicarbonate or water.

Toxicity curves made by the intravenous injection of the acetates of strontium and calcium into 280 rats and 140 mice show that the greater effect of strontium on the uterus is not accompanied by a correspondingly greater increase in general toxicity.

**Phosphorylation of Glucose in Kidney Extract.** BY SIDNEY P. COLOWICK, MARY S. WELCH, AND CARL F. CORI. *From the Department of Pharmacology, Washington University School of Medicine, St. Louis*

Kalckar\* has shown that fructosediphosphate accumulates when glucose, inorganic phosphate, and fluoride are added to kidney extract and that this phosphorylation occurs under aerobic conditions only. He also found that addition of citrate, glutamate, or succinate increased the rate of respiration and phosphorylation. The formation of fructosediphosphate was confirmed; in some cases phosphoglyceric acid was also present among the products of glucose phosphorylation. In order to study the components of this system kidney extracts were aged for 24 hours at 10° or they were dialyzed for 4 hours at 0°.

Aged extracts respired at less than one-half of the original rate and did not phosphorylate glucose. Addition of 0.001 M. adenylic acid restored both respiration and phosphorylation to the original level. In some aged extracts phosphorylation was restored only when both adenylic acid and cozymase were added, showing that both are necessary components of the system.

Dialyzed extracts neither respired nor phosphorylated glucose and were not restored by addition of adenylic acid and cozymase. When M/40 citrate, l(+)-glutamate, ketoglutarate, or succinate was added, there was some increase in respiration but no phosphorylation of glucose. When in addition to these substrates  $Mg^{++}$  was added, respiration increased 2 to 5 times and large amounts of glucose were phosphorylated. Malate or pyruvate

\* Kalckar, H., *Enzymologia*, 6, 209 (1939).

plus  $Mg^{++}$  did not cause phosphorylation in extracts which were reactivated by succinic acid. This suggests that the phosphorylation of glucose in this system may be linked with the dehydrogenation of succinic to fumaric acid.

**Preparation of Keto-1-Uronic Acids and Their Relations to Carbohydrate Oxidation.** BY L. T. CREWS, FAY SHEPPARD, H. C. SUDDUTH, AND MARK R. EVERETT. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

A series of keto-1-uronic (osonic) acids has been prepared by an improved method based upon cupric acetate oxidation of aldoses in alcoholic solution, bromine oxidation of the resulting osones, separation of the desired acids as barium salts, and crystallization of pure alkaloidal salts. New acids isolated by this procedure will be described.

Studies of the relations of keturonic acids to salt-catalyzed hydrogen peroxide oxidation of carbohydrates have provided information concerning the oxidative mechanism. Pentoses constitute approximately two-thirds of the accumulating reducing products from monocarboxylic acids in this reaction, owing to the stability of aldoses (and ketoses) to hydrogen peroxide. The remaining reducing substances are keturonic acids stable to bromine and precipitable as barium salts. Only small quantities of keto-5-uronic and keto-6-uronic acids are formed and these important secondary products of bromine oxidation of aldoses are not active intermediates in hydrogen peroxide oxidation of monocarboxylic acids. *l*-Sorbo-6-uronic acid requires bromine for salt-catalyzed oxidation but *l*-sorbo-1-uronic acid is actively oxidized by hydrogen peroxide under similar circumstances. Since no *d*-arabinose is formed from *l*-sorbo-1-uronic acid, the pentoses originate by an interesting independent oxidative decarboxylation of monocarboxylic acids in which keto-1-uronic acids are not intermediates. However, these acids are significant intermediates for more extensive degradation of carbohydrates by hydrogen peroxide. Monocarboxylic and keto-1-uronic acids are attacked chiefly at carbon atoms 1 and 2. Polyhydric alcohols are also oxidized by hydrogen peroxide with formation of ketoses and other reducing substances.

**Factors Controlling Muscle Water and Electrolyte.** BY DANIEL C. DARROW, HERMAN YANNET, AND HERBERT C. MILLER.  
*From the Department of Pediatrics, Yale University School of Medicine, New Haven*

Cats were subjected to loss of body sodium and chloride and the muscle and serum analyzed for water and the principal anions and cations. Chloride of the muscle varies directly with the concentration in serum, as if practically all muscle chloride were present in an ultrafiltrate of plasma. Muscle sodium varies directly with the concentration of serum sodium but a larger volume of fluid is necessary to contain muscle sodium at the concentration of an ultrafiltrate of plasma than that necessary for chloride. Variations in tissue water are largely intracellular and tissue water varies inversely with the concentration of sodium in serum. The relationship is such that when the concentration of sodium in serum is reduced, the concentration of univalent base in intracellular water decreases only about two-thirds as much as does the concentration of univalent base in serum.

Experiments on rats show that tissue potassium varies inversely with intracellular sodium. When muscle potassium is normal, it is difficult to raise the tissue concentration but, if a rise is produced, there is evidence of loss of muscle sodium. When muscle potassium is abnormally low, injected potassium is readily retained by the muscle. Evidence is available that the factors controlling the distribution of muscle water and electrolyte are the same in adrenalectomized rats and rats with adrenals intact.

**The Colorimetric Determination of Cholic Acid.** BY ROBERT T. DILLON. *From G. D. Searle and Company, Chicago*

The colorimetric determination of cholic acid is an important analysis for those dealing with bile and bile acids. The current generally accepted modification of the Pettenkofer reaction (now known as the Gregory-Pascoe modification) is that of Reinhold and Wilson.\* Doubilet more recently has contributed additional worth while changes.

However, throughout the development of this analytical procedure numerous questions have been left unsolved, others are seemingly inconclusively demonstrated, and in some few cases

\* Reinhold, J. G., and Wilson, D. W., *J. Biol. Chem.* 96, 637 (1932).

contradictory remarks appear. This leaves a valuable colorimetric determination in an unsatisfactory and confused state. The factors may be divided into those which concern the method of preparing the bile solutions preparatory to colorimetric analysis and those which concern the various effects on the colorimetric determination itself. In the first group belong the protein precipitation procedures with alcohol and with zinc hydroxide and other preanalytical treatment of the sample, such as alcohol and alkali treatment. Studies have been carried out on these effects in both pure cholic acid and bile solutions so that more specific directions for procedure can be outlined. In the last group belong the effects of alcohol and salts upon the actual colorimetric determination of cholic acid, as well as other factors in the technique of determination, such as original color in the bile solutions, stability of the furfural solutions, and the effect of alcohol and acetic acid addition to the final colorimetric solutions. Studies on these factors have cleared up several questions.

**The Determination of Ketone Groups in Ketocholanic Acids.** BY ROBERT T. DILLON AND ALBERT L. RAYMOND. *From G. D. Searle and Company, Chicago*

Many procedures are described for analyzing for the ketone groups in organic compounds but the results attainable with the various modifications are inconsistent and inaccurate. The usual reagent is hydroxylamine, which readily reacts with the ketone groups present. Bryant and Smith\* describe a modification of the usual procedure by adding pyridine to the reaction mixture, which apparently brings about more favorable equilibrium conditions for the reaction and may perhaps catalyze it. The usefulness of this modification in synthetic work has been amply demonstrated, chiefly on semicarbazone formation, but as an analytical procedure it is, by the very nature of the method of titration, very limited in use.

For the analysis of ketocholanic acids we have used the same reaction of hydroxylamine in alcoholic pyridine solution. The reaction of the ketone groups with hydroxylamine is carried out at 100° in pressure bottles. Titration of the final mixture, along with a suitable reagent blank, is conveniently carried out in 15

\* Bryant and Smith, *J. Am. Chem. Soc.*, 57, 57 (1935).

per cent alcohol solution in which the oximes of the ketocholanic acids are insoluble. Titration with 0.5 N hydrochloric acid solution is carried to a suitable end-point of about pH 3.0, with a glass electrode indicating system. An accuracy of better than 1 per cent is easily attained with ketocholanic acids such as dehydro-lithocholic acid, dehydrodesoxycholic acid, dehydrochenodesoxycholic acid, dehydrocholic acid, and other keto derivatives of cholanic acid.

Other modifications of the hydroxylamine reaction were tried but the results were not satisfactory.

### Concerning the Metabolism of Testosterone to Androsterone.

By RALPH I. DORFMAN AND JAMES B. HAMILTON. *From the Adolescence Study Unit, the Laboratory of Physiological Chemistry, and the Department of Anatomy, Yale University School of Medicine, New Haven*

After administration of the testis hormone, testosterone, to men with deficient testicular secretions the principal androgen excreted in the urine is androsterone.\* In order to study the mechanism of this conversion we have investigated the metabolism in the human of testosterone, methyltestosterone, androsterone, dehydroisoandrosterone, and three theoretical intermediates; namely, etioallocholan-3( $\alpha$ )-17-diol, etioallocholan-3,17-dione, and etiocholen-(4,5)-3,17-dione.

The compounds were administered orally except in the case of testosterone which was administered both orally and intramuscularly. The subjects were four men, three showing definite symptoms of hypogonadism and the fourth a surgical castrate. The complete output of urine was collected during the periods of treatment which varied from 6 to 10 days. The urines were hydrolyzed with hydrochloric acid and extracted with benzene. The neutral compounds of the benzene extract were separated into the ketonic and non-ketonic fractions by means of the Girard-Sandulesco reagent. Each fraction was assayed for androgenic activity. It was found that all of the compounds administered were absorbed from the gastrointestinal tract, as measured by increased excretion of androgenic substances in the urine. This

\* Callow, N. H., *Biochem. J.*, **33**, 559 (1939). Dorfman, R. I., Cook, J. W., and Hamilton, J. B., *J. Biol. Chem.*, **130**, 285 (1939).

increase was especially marked in the fraction containing the ketonic substances.

Androsterone was isolated from ketonic fractions of the urines collected after the administration of testosterone, androsterone, etioallocholan-3,17-dione, and etiocholen(4,5)-3,17-dione. The isolation of androsterone after the administration of the latter two compounds is particularly interesting, since they may well be intermediates in the conversion of the testis hormone, testosterone, to the urinary androgen, androsterone.

### **Intravenous Fat Is Not a Precursor of Cholic Acid in the Dog.**

BY MILDRED E. DOSTER-VIRTUE AND ROBERT W. VIRTUE.

*From the Department of Chemistry, University of Denver, Denver*

Bile fistula dogs were maintained for consecutive 3 day periods on a diet of carbohydrate, on intravenously injected homogenized fat emulsions, and on a protein diet. Each type of material furnished the animals 50 calories per kilo per day. The cholic acid excretion on the carbohydrate and fat régimes decreased steadily, whereas on the protein diet it rose to high values.

### **6( $\alpha$ )-Hydroxyprogesterone.** BY MAXIMILIAN EHRENSTEIN AND THELMA O. STEVENS. *From the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia*

The preparation of progesterones and desoxycorticosterones which are hydroxylated at various carbon atoms of the sterol nucleus appears desirable in order to investigate the chemical specificity of "corpus luteum hormone" and "cortin" action. The acetate of the 6( $\alpha$ )-hydroxyprogesterone was obtained by means of the following procedure: 5-pregnen-20-on-3-ol yielded with hydrogen peroxide pregnan-20-on-3( $\beta$ )-5,6(*trans*)-triol; m.p. 256–258°.\* Acetylation of the latter furnished the 3,6-diacetate; m.p. 215.5–216.5°. With special precautions, a partial saponification was performed whereby the hydroxyl group at carbon atom 3 was set free. Melting point of the 6-monoacetate, 222–226°. Oxidation of the latter compound with chromium trioxide furnished pregnane-3,20-dione-5,6(*trans*)-diol 6-monoacetate; m.p. 215–217.5°. This compound was dehydrated by means of dry hydrochloric acid in a solution of chloroform. Thereby 4-preg-

\* See also Ehrenstein, M., *J. Org. Chem.*, **4**, 506 (1939).

nene-3,20-dion-6( $\alpha$ )-ol acetate (6( $\alpha$ )-hydroxyprogesterone acetate) was obtained; m.p. 145–146°. Saponification to the free 6( $\alpha$ )-hydroxyprogesterone is under way. Pregnan-20-on-3( $\beta$ )-5,6(*cis*) is being used to prepare 6( $\beta$ )-hydroxyprogesterone.

**The Distribution of Body Water in Skeletal Muscle in Normal Dogs Following Injections of Potassium Salts.** BY LILLIAN EICHELBERGER. *From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago*

Data will be presented on volumes of the extracellular and intracellular phases of skeletal muscle of normal dogs, both before and after increases in potassium and total body water produced by the intravenous injection of an isotonic solution containing 25 mM  $\text{KHCO}_3$  + 129 mM  $\text{NaCl}$ . These findings will be compared statistically with those obtained both previous to and following the intravenous injection of a solution containing 25 mM  $\text{NaHCO}_3$  + 129 mM  $\text{NaCl}$  on normal animals serving as controls. All data are expressed in terms of fat-free, blood-free muscle.

Following the increase in body potassium and total body water, the total bulk of the skeletal muscle increased 69 gm. per kilo of original muscle as compared with an average of 58 gm. following the sodium chloride-sodium bicarbonate injections. This increase, as in the case of  $\text{NaCl}$ - $\text{NaHCO}_3$  injections, is entirely accounted for by the hydration of the extracellular phase, the intracellular phase remaining unchanged. From these findings, it is concluded that in the normal organism, after a simultaneous increase in body potassium and total body water, the additional water is distributed in the same way as the increased body water resulting from the intravenous injection of the isotonic sodium chloride-sodium bicarbonate solution. Therefore, in these experiments there is no indication of any influence of potassium upon the distribution of fluid in skeletal muscle in the normal organism.

**A Study of the Permeability of the Human Erythrocytes to Potassium, Sodium, and Phosphate by the Use of Radioactive Isotopes.** BY ANNA J. EISENMAN, L. OTT, PAUL K. SMITH, AND ALEXANDER W. WINKLER. *From the Department of Internal Medicine and the Laboratory of Pharmacology and Toxicology, Yale University School of Medicine, New Haven*

Transfers of potassium, sodium, and phosphate between human serum and red cells were studied by the use of radioactive isotopes (furnished through the cooperation of Dr. Ernest C. Pollard of the Department of Physics). After 4 hours at 38° very little if any radiosodium and radiopotassium entered the cells. There were also no significant transfers of sodium and potassium as determined chemically. Therefore potassium and sodium in the cells were not in free diffusion equilibrium with the potassium and sodium outside the cells.

As much as 40 per cent of the radiophosphorus entered the cells, although the net transfer of inorganic phosphate, determined chemically, was much smaller. This is evidence that there is active entry of radiophosphorus and a subsequent synthesis of this into organic phosphates. Since the total inorganic phosphates did not change, there must have been a simultaneous breakdown of esters to inorganic phosphate. After 4 hours at 7° very little transfer either of radiophosphorus or of inorganic phosphates could be detected. Transfer of phosphate across the cell membranes is thus associated with enzymatic processes and not with passive diffusion.

**Chemical Determination of Thiamine by the Melnick-Field Method.** BY A. D. EMMETT, GAIL PEACOCK, AND RAYMOND A. BROWN. *From the Research Laboratories of Parke, Davis and Company, Detroit*

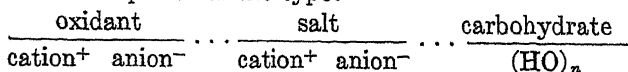
Using the Prebluda-McCollum reagent (*p*-aminoacetophenone) for vitamin B<sub>1</sub>, together with xylene, alcohol, and phenol as recommended by Melnick and Field, we were able to confirm their findings that the method gives quantitative results. We substituted the Lovibond tintometer for the colorimeter. This permitted a wider range of color comparison. The results for thiamine solutions, extracts of wheat germ, yeast, and liver compared favorably with the bioassay values. Further, we found that nicotinic acid did not interfere, whereas vitamin C did, unless it was first oxidized by titrating with iodine.

**Salt Catalysis of Carbohydrate Oxidation.** BY MARK R. EVERETT AND FAY SHEPPARD. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*  
Published studies of carbohydrate oxidation in aqueous solution



have dealt largely with degradation products of slow reactions at alkalinities which favor fission and dismutation. Our quantitative investigations of keturonic acid formation provide direct information concerning primary phases of carbohydrate oxidation. Comprehensive studies of the effects of salts (including compounds of 57 elements and of numerous organic acids) demonstrate that certain salts are very active catalysts for oxidation of carbohydrates by bromine or hydrogen peroxide at pH zones near neutrality. Certain synergistic and antagonistic activities of ions in these reactions resemble ionic balances known to affect biological carbohydrate oxidation and the syntheses dependent upon energy from this source. Hydrogen peroxide oxidation is catalyzed by relatively few of the salts effective with bromine; of these, alkali bicarbonates and copper and iron salts have especial biological significance.

The oxidative catalytic activities of salts are more specifically related to the type of oxidant than to the carbohydrate substrate. Cations are the effective catalytic components of the salts and they usually exhibit their activities only in association with certain anions. However, anionic components of copper and iron salts tend to be less important for catalysis of oxidation by hydrogen peroxide. There is evidence that some of the most effective anions (tungstate, stannate, molybdate, borate, pyroantimonate, bicarbonate, hydroxide, and titanate) form inactive carbohydro-anion complexes which are activated by combination with appropriate cations. Salt catalysis evidently operates through coordination complexes of the type:



**Effect of Various Endocrine Extracts upon the Amino Acids in the Blood of Dogs.** BY LEE E. FARR AND LOUIS K. ALPERT.  
*From the Hospital of The Rockefeller Institute for Medical Research, New York*

The discovery by Farr and MacFadyen of a chronic amino acid deficit in the blood plasma of nephrotic children and of acute exacerbations of this deficit during nephrotic crises directed attention to a search for mechanisms controlling the level of blood amino acids. Using Van Slyke, Dillon, and MacFadyen's\* specific

\* Van Slyke, Dillon, and MacFadyen, unpublished work.

ninhydrin method for determining blood amino acid content, we confirmed previous investigators' observations that epinephrine and insulin given subcutaneously decrease the plasma amino acids. On the other hand, pitressin, antuitrin-S, adrenal cortical hormone, testosterone propionate, and thyroxine injected intravenously increase the plasma amino acids. Growth-promoting substance of the anterior pituitary shows a peculiar behavior: intraperitoneally it decreases plasma amino acids, but intravenously it raises them. None of the amino acid changes was accompanied by significant changes in blood urea nitrogen. Changes in the plasma amino acids after insulin were not accompanied by corresponding changes in cell amino acids.

**The Microbiological Determination of Riboflavin.** BY R. E. FEENEY AND F. M. STRONG. *From the Department of Biochemistry, University of Wisconsin, Madison*

Evidence that the microbiological assay of Snell and Strong measures not only free riboflavin but also combined forms was obtained by successful assays of Warburg's flavin-adenine dinucleotide and two samples of milk flavoprotein. The addition of relatively large amounts of vitamin B<sub>6</sub>, pantothenic acid, nicotinic acid, factor W, biotin, cocarboxylase, cozymase, and adenylic acid to the basal medium had no significant effect on the assay of pure riboflavin.

Investigations of experimental conditions involved in various analytical procedures for riboflavin showed that the following may cause serious destruction as measured by the bacteriological assay: exposure to diffuse daylight for more than a few minutes, autoclaving at a pH above 8.0, autoclaving with strong acids more concentrated than 0.1 N, oxidation with KMnO<sub>4</sub> in acetic acid solution, refluxing with H<sub>2</sub>O<sub>2</sub> in N H<sub>2</sub>SO<sub>4</sub>-acetone (1:3) solution. Dry materials containing riboflavin were stored in the dark at room temperature for extended periods without appreciable losses.

The method has been extended with fair success to a number of human and animal bloods, and with entire success to urine, feces, Lloyd's reagent carrying adsorbed riboflavin, and a variety of other materials.

**Gastric Emptying and Intestinal Absorption of Glucose Solutions in the Rat.** BY P. F. FENTON AND H. B. PIERCE. *From the*

*Department of Physiological Chemistry, College of Medicine,  
University of Vermont, Burlington*

Glucose solution of known volume, pH, and concentration was fed to fasted adult rats by stomach tube. At the end of a definite time interval (15, 30, 45, or 60 minutes) the animals were sacrificed, the abdomen slit open, and ligatures placed at the cardia, pylorus, and ileocecal sphincter. The stomach and small intestine were removed and their glucose, chloride, and fluid contents determined by suitable chemical methods. The pH of the gastric fluid was determined with a micro glass electrode. From the known concentrations and volumes of solution at the beginning and end of the experimental period, the volume of gastric secretion, the volume of fluid and amount of glucose emptied from the stomach, and the amount of glucose absorbed were calculated.

The volume of gastric secretion was found to be almost identical for isotonic and 64 per cent glucose solutions; however, the pH of gastric fluid in the latter case was distinctly higher, indicating an inhibition of secretion of gastric juice and an augmented secretion of non-acid fluid, probably water and mucus.

The volume of fluid emptied per unit time was less with 64 per cent glucose than with isotonic solution, although more glucose was emptied with the former owing to the much greater concentration of the test solution.

The amount of sugar emptied and the amount absorbed increased with the concentration of the test solution. Furthermore, the rate of emptying and the rate of absorption were not linear functions but rather decreased markedly during the time interval studied.

#### **Muscle Glycogen Resynthesis after Exercise.** BY EUNICE V.

FLOCK AND JESSE L. BOLLMAN. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

Muscles of one hind leg of rats anesthetized with nembutal were stimulated directly to contract isotonically three times each second. Analysis of the muscles of each leg at subsequent intervals showed a rapid disappearance of glycogen, 60 to 80 per cent being lost within the first 3 minutes. Only minor changes of the glycogen content occurred with subsequent continuous work. With rest after work the high concentrations of lactic acid and hexosemonophosphate in the muscle return to normal before an

increase in the low glycogen content can be detected. After 30 minutes rest a definite increase of glycogen is found and normal values are approximated after 1 to 3 hours. The rate of glycogen resynthesis is not materially altered by variations in the glucose content of the blood produced by hepatectomy, insulin, adrenalin, or by glucose administration. There is some delay in the rate of recovery after a fast of 48 hours.

In rats poisoned with iodoacetate, cyanide, or azide, or when the muscle is deprived of its blood supply contraction ceases after the 1st minute of stimulation. The changes in the muscle constituents are similar to those found after corresponding work in normal animals. With rest after work no change in glycogen occurs and the lactic acid content of the muscle may remain elevated. The glycogen content of the resting leg is surprisingly little affected in the first 30 minutes.

**Notes on Gasometric Plasma Lipid Analyses.** BY JORDI FOLCH, H. A. SCHNEIDER, AND DONALD D. VAN SLYKE. *From the Hospital of The Rockefeller Institute for Medical Research, New York*

*Free Cholesterol*—When petroleum ether solutions of plasma lipids are taken to dryness, as in precipitation of cholesterol digitonide in the procedure of Kirk, Page, and Van Slyke,\* a slight amount of other organic matter in the extract becomes insoluble in both the ether and hot water used for washing the precipitate. In consequence the precipitate, when measured by its carbon content, gives results about 20 per cent too high. This error does not affect the total cholesterol estimation; apparently saponification alters the contaminant so that it no longer interferes. The error in free cholesterol is avoided by precipitating the digitonide in a medium containing water, absolute alcohol, and ether in the proportions 2:9:9. The precipitate forms quantitatively without taking to dryness when the mixture is kept overnight in an ice box.

*Lipid Amino Nitrogen*—In the plasma lipids purified as recently described,† a large part of the  $N_2$  set free by reaction with  $HNO_2$

\* Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 203 (1934).

† Folch, J., and Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, **41**, 514 (1939).

under conditions used for lipid  $\text{NH}_2$  determination\* is due to the unsaturated groups in the fatty acids, one unsaturated linking evolving from  $\text{HNO}_2$  about 0.12 mole of  $\text{N}_2$ . When saturated as completely as possible in ether solution with  $\text{H}_2$  in the presence of Pt catalyst, the total lipid mixture from human plasma yields with  $\text{HNO}_2$  only 0.02 to 0.08 mole of  $\text{N}_2$  per atom of phosphorus present, indicating but little free amino nitrogen in the phosphatides. Short hydrolysis with  $\text{HCl}$  sets free a water-soluble nitrogen compound, which reacts with  $\text{HNO}_2$  only after further prolonged hydrolysis.

**Seasonal Variation in the Metabolism of the Pneumococcus.** By THEODORE E. FRIEDEMANN. *From the Laboratory of Chemical Bacteriology, the Department of Medicine of the University of Chicago, Chicago*

An examination of metabolic data obtained over a period of several years, from 1930 to 1937, reveals a marked seasonal variation in the intermediary metabolism of carbohydrate. The maximum yield of 95 per cent of lactic acid is attained in July and August. Traces of volatile products are also produced. The yield of lactic acid then diminishes gradually until a minimum of about 45 per cent is reached in January and February. Large quantities of formic acid, acetic acid, and ethyl alcohol, produced in the ratio of 2:1:1, are present in the culture medium at this time.

The culture medium was prepared from fresh beef muscle just before each experiment. These data for the first time demonstrate a difference in the composition of such culture media which is sufficiently great to affect the metabolism of bacteria. Marked variations in the intermediary metabolism are probably accompanied by deep seated changes in other functions. A study of such variations, especially as related to the many seasonal changes observed in animals, may throw light upon the perplexing problem of the seasonal occurrence of certain infectious diseases.

**The Specificity of Proteolytic Enzymes from Normal and Tumor Tissues.** By JOSEPH S. FRUTON. *From the Laboratories of The Rockefeller Institute for Medical Research, New York*

The availability of substrates of known structure and configuration has made possible a precise study of the specificity and

activation phenomena of the intracellular proteolytic enzymes. Partially purified extracts of beef spleen and beef and swine kidney have been found to hydrolyze an extensive series of synthetic peptides and peptide derivatives. In their action on most of these substrates, the enzyme preparations are decisively activated by the addition of substances such as cysteine, glutathione, the ascorbic acids, or HCN. From these studies it appears that in spleen and kidney extracts there are at least three types of proteolytic activity that may be characterized by their structural specificity and activation behavior.

Enzyme preparations from six tumors (Brown-Pearce rabbit carcinoma, Bashford mouse carcinoma, mouse Sarcoma M-180, sarcoma of human bone, and carcinomas of human breast and thyroid) also hydrolyze a number of the synthetic substrates; the enzymatic action of these extracts is usually less than that observed with extracts of the normal tissues.

In the case of two tumor extracts it was noted that peptide linkages involving *d*-amino acids (*e.g.* carbobenzyloxy-*d*-glutamyl-*l*-tyrosine or *d*-leucineamide) were hydrolyzed at rates similar to those for the corresponding *l* forms. Extracts of the normal tissues as well as the other tumors hydrolyzed the *l* form of these substrates much more rapidly than the corresponding *d* forms.

**Effects of Anterior Pituitary Preparations in Experimental Pancreatic Diabetes.** BY OLIVER HENRY GAEBLER AND HARRY W. GALBRAITH. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

This study is concerned with acute effects of single injections of anterior pituitary preparations, not with the permanent glycosuria produced in intact animals by prolonged injections. It concludes a series of studies in which the same preparation, in the same dose, was administered to normal and phlorhizinized dogs, and to depancreatized dogs maintained by diet and insulin.

In normal animals no definite diabetogenic effects were observed; on the contrary, the nitrogen storage reported in earlier experiments was obtained. In depancreatized dogs the same dose produced marked glycosuria, ketonuria, hyperglycemia, and lipemia. It was necessary to double the maintenance dose of insulin, and the increased insulin requirement persisted for a month or more.

In meat-fed phlorhizinized dogs the same dose increased the ketonuria, but both nitrogen and glucose output were lower than in controls receiving phlorhizin alone, and the D:N ratio was 3.3. There was thus no evidence for increased conversion of protein or fat to glucose. In depancreatized dogs, if the injection of the growth preparation was accompanied by doubling of the insulin intake, the hyperglycemia and glycosuria were still produced, although the nitrogen output was diminished markedly, thus again eliminating protein as the source of the sugar. Interference with carbohydrate oxidation, and perhaps glycogen loss, remain as the most plausible explanations of the acute effects in depancreatized dogs under our conditions.

**The Oxidation of Bile Acid Esters by Aluminum Isopropylate and Acetone.** BY T. F. GALLAGHER. *From the Department of Biochemistry of the University of Chicago, Chicago*

The oxidation of the esters of bile acids (cholic, desoxycholic, and 3-hydroxy- $\Delta$ -5-cholenic) by means of aluminum isopropylate in the presence of acetone and other ketones has been studied. The reaction appears to attain equilibrium with the production of compounds in which only the hydroxyl group at carbon atom 3 is oxidized. The ethyl esters of cholic acid and 3-ketocholic acid form a series of mixed crystals and probably a 1:1 molecular compound. The oxidation of other steroids is being investigated in order to determine whether the hydroxyl at carbon atom 3 is specifically oxidized. The biological properties of 3-ketocholic acid have been studied.

**The Oxidation of Vitamin E.** BY CALVIN GOLUMBIC. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

$\alpha$ -Tocoquinone, the first oxidation product of  $\alpha$ -tocopherol which can be isolated, possesses no vitamin E activity. The same is true of the related  $\alpha$ -tocohydroquinone and  $\alpha$ -tocohydroquinone triacetate. The biological significance of these results is discussed in relation to the several reversible oxidation-reduction systems produced by *in vitro* oxidation of  $\alpha$ -tocopherol. On the assumption of the existence of a tocosemiquinone, three of such systems are possible.

An approximation of the "apparent oxidation potential" of

synthetic  $\alpha$ -tocopherol was secured by oxidation of the compound with benzoquinone and methylquinones followed by electrometric analysis of the product for unchanged tocopherol. By this method, the "apparent oxidation potential" was found to lie in the range of a mono- or dimethylhydroquinone. Benzohydroquinones or benzoquinones with oxidation-reduction potentials in this region did not function as vitamin E. Properties other than the mere possession of an oxidation-reduction potential in the proper range are requisite for vitamin E activity of a substance.

**Intestinal Absorption of Vitamin A in the Normal Rat.** BY E. LeB. GRAY, KENNETH MORGAREIDGE, AND JOHN D. CAWLEY. *From the Laboratories of Distillation Products, Inc., and the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Evidence is accumulating that indicates a higher conversion factor for vitamin A esters than for the alcohol, which has led to some speculation concerning the possibility that vitamin A esters may be absorbed as such without hydrolysis to the alcohol. Thirty-six adult female rats were each given by stomach tube a single dose of 54,000 U.S.P. units of distilled vitamin A ester in 1.0 cc. of corn oil. At regular intervals up to 400 minutes, groups of four rats were etherized and the gastrointestinal tract from the esophagus to the ileocecal junction ligated and removed intact. The stomach and gut contents were washed out with saline and total lipid extracts of both washings and gut wall (including the stomach) prepared in the usual manner. Total vitamin A of the extracts was determined. Recoveries of the dose fed ranged from 40 to 60 per cent. The samples were then distilled by analytical technique to determine the ratio of vitamin A esters to alcohol. It was found that the per cent of alcohol in the gut increased steadily from 4.4 per cent at 80 minutes to 16.2 per cent at 400 minutes, while the alcohol in the gut wall increased from 59.2 per cent at 220 minutes to 81.9 per cent at 400 minutes. The alcohol content of the original preparation was less than 1 per cent. These results indicate that vitamin A esters are hydrolyzed in the gut prior to absorption, and that during the height of absorption the vitamin exists in the gut wall chiefly as the alcohol.



**Factors Concerned in the Development of Tetany by the Rat.** By

DAVID M. GREENBERG, MURIEL D. D. BOELTER, AND BENJAMIN W. KNOPF. *From the Division of Biochemistry, University of California Medical School, Berkeley*

A study has been made of the relation of certain dietary and hormonal factors to the production of tetany in the rat.

Extreme lack of calcium in the diet, alone, does not produce tetany, even though the blood calcium drops to extremely low levels. Tetany will result if the diet is deficient both in vitamin D and calcium.

On the low calcium diet, tetany is produced by removal of the parathyroids and even more effectively by removal of the thyroid with the parathyroids.

The attacks of tetany produced by the different procedures pursue a like course but show a difference in response to different stimuli. Attacks can be induced with a galvanic shock but not with the sound of an air blast in tetany associated with vitamin D and calcium deficiency or with healing rickets. Both stimuli are effective in tetany due to thyroparathyroidectomy, and the air blast only is an effective stimulus in the tetany of magnesium deficiency.

Involvement of the central nervous system in the syndrome of tetany is shown by the following: (a) The rats are more sensitive to the mid-brain drug picrotoxin, (b) sedative doses of amytal and pentobarbital prevent the onset of attacks, and (c) rats with spinal transection reacted with the fore body but not with the hind extremities in attacks.

**Effect of Salts on the Physical Properties of Sodium Thymonucleate.** By JESSE P. GREENSTEIN. *From the National Cancer Institute, United States Public Health Service, Bethesda, Maryland*

Aqueous solutions of sodium thymonucleate are highly viscous and possess intense double refraction of flow. These properties are related to the extreme asymmetry of the highly polymerized nucleic acid molecules.

The effect on these properties of a large number of inorganic and organic salts over a wide concentration range has been investigated. Protein-free, native sodium thymonucleate (Hammarsten)

was used. Certain of the results may be summarized as follows: (1) All of the salts studied markedly reduce the viscosity and either diminish or destroy the streaming birefringence, the extent of the effect depending upon the nature and the concentration of the salt; (2) the most effective cation in this regard is guanidonium and the most effective anions are iodide and thiocyanate; (3) coincident with the drop in viscosity and loss of double refraction, the solution of salt and nucleic acid becomes isotropic and no longer light-scattering; (4) the effect is apparently completely reversible; *i.e.*, removal of the salt restores the original viscosity, light scattering, and intensity of birefringence.

### The Sulfhydryl Groups of Rabbit and Calf Liver Nucleoprotein.

By JESSE P. GREENSTEIN. *From the National Cancer Institute, United States Public Health Service, Bethesda, Maryland*

A nucleoprotein fraction from rabbit and from calf liver has been isolated by a procedure based upon repeated isoelectric precipitation in the presence of 0.8 M salt, followed each time by thorough washing at the centrifuge with 0.8 M salt. Six precipitations were performed; after the third a constant N:P ratio was obtained. All procedures were conducted at 5°. The protein freed of salt was dried under high vacuum at low temperature. The yield was consistently 10 gm. from 2 kilos of wet tissue.

Four preparations of rabbit protein yielded the following average values, N 15.8, P 0.71, amide N 1.0, S 0.84. Two preparations of calf protein yielded N 14.9, P 0.82, S 0.95.

All the preparations gave a strong nitroprusside reaction. The free —SH groups of the proteins in the native and denatured states were estimated by porphyrindin titration and expressed as cysteine. For native rabbit protein a value of 1.4 per cent cysteine was obtained; after denaturation with guanidine hydrochloride exactly the same value was obtained. Native calf liver protein gave a value of 0.6 per cent cysteine; after denaturation the value rose to 0.8 per cent. Apparently in the rabbit protein, in contrast to the calf, there are no "masked" sulfhydryl groups—all of the groups are titratable in the native state. Cysteine accounts for half the total sulfur in the rabbit protein and for approximately one-fourth in the calf protein.

**A Crystalline Protein from Normal Human Urine.** BY MARTIN E. HANKE. *From the Department of Biochemistry of the University of Chicago, Chicago*

About 50 mg. of protein per liter of normal urine are precipitated by addition of 1 per cent ammonium sulfate and several hours standing. The material is collected by centrifugation, washed twice with 1.5 per cent ammonium sulfate, extracted with water, and precipitated by adding alcohol to 65 per cent. Further purification can be effected by extraction with water, and reprecipitation with 65 or 80 per cent alcohol, in the presence of 2 or 0.5 mg. of NaCl per cc., respectively. As previously reported,\* these preparations, in a 1 mg. dose, markedly inhibit gastric secretion and motility in dogs. Biuret, the glyoxylic acid test for tryptophane, and the Millon test for tyrosine are positive. The protein is not heat-coagulable, is non-dialyzable through cellophane, and gives a strong Molisch test.

When this crude protein preparation is dissolved in 33 per cent of saturated ammonium sulfate and gradually in 1 day is brought to 35 and finally in 2 more days to 38 per cent of saturated ammonium sulfate, microscopic needles separate, which, after removal of the mother liquor, may be recrystallized. The yield of crystals is about 5 per cent of the crude protein, or 2 mg. per liter of urine. Further increase in the ammonium sulfate concentration causes amorphous precipitation of the bulk of the protein. As distinct from the crude protein, the crystals give a negative Molisch test, and show about half as much gastric inhibitory activity. Biuret, glyoxylic acid, and Millon reactions are strongly positive.

**The Rate of Turnover of the Lecithins and Cephalins of Rat Carcinosa 256 As Measured by Radioactive Phosphorus.**

BY FRANCES L. HAVEN.\* *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

By the use of radioactive phosphorus it has previously been shown that in their rate of turnover the phospholipids of rat

\* Necheles, H., Hanke, M. E., and Fantl, E., *Proc. Soc. Exp. Biol. and Med.*, **42**, 618 (1939).

\* Fellow of the National Cancer Institute.

Carcinosarcoma 256 resemble those of liver rather than those of muscle. The rate of turnover of the lecithin fraction of this tumor has been found to be more rapid than that of the cephalin fraction.

Rats bearing Carcinosarcoma 256 were killed from 4 hours to 26 days after receiving by stomach tube a solution of disodium hydrogen phosphate containing radioactive phosphorus. Tumor phospholipids were isolated and the lecithin and cephalin fractions separated by means of absolute alcohol. The degree of separation was measured by choline determinations on the total mixed phospholipid and on the lecithin fractions. The radioactivity, expressed as percentage of dose per gm. of phospholipid, was significantly greater in the lecithin fraction than in the cephalin fraction from 4 through 30 hours after feeding. The activities then became about the same and decreased at approximately the same rate. This is indicative of a further resemblance between the phospholipids of this tumor and those of liver, since the lecithin fraction of rat liver has been found (Chargaff) to possess greater activity than the cephalin fraction 24 hours after administration of radioactive phosphorus, while the activities of these fractions obtained from the carcass were approximately the same.

**Studies on Additional Factors Required by the Chick.** BY D.

MARK HEGSTED, J. J. OLESON, C. A. ELVEHJEM, AND E. B. HART. *From the Department of Biochemistry, University of Wisconsin, Madison*

Using a simplified diet, adequate in the vitamin B complex, we have demonstrated the existence of an essential growth factor for chicks, present in cartilage, kidney, and rice and distinct from the anti-gizzard erosion factor.\* Cartilage, which is low in the B vitamins, can be advantageously used as a source of this growth factor and the gizzard factor in purified diets for the study of the B complex requirements of the chick.

The diet used consists of dextrin 57 per cent, casein 18, cartilage 15, salts 5, soy bean oil 5, and vitamins A and D concentrate. 2 mg. per kilo of thiamine and riboflavin are added. When this diet, supplemented with 3 per cent alcohol-soluble liver extract, is fed, poor growth is obtained unless crystalline vitamin B<sub>6</sub> is added,

\* Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B, *J. Biol. Chem.*, **130**, 423 (1939).

showing the need for this vitamin. If supplements of a fullers' earth filtrate of liver extract and vitamin B<sub>6</sub> are used, optimum growth results only if a factor U concentrate, prepared from yeast, is added. In spite of this improved growth, the chicks develop severe dermatitis, similar to that observed in pantothenic acid deficiency; even if high levels of pantothenic acid are added. This dermatitis is prevented by yeast or yeast residue remaining after factor U extraction. The properties and distribution of this factor indicate that it may be the same as the anti-egg white injury factor (vitamin H).

**Some Properties of "Angiotonin."** BY O. M. HELMER AND IRVINE H. PAGE. *From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis*

Recently Page and Helmer\* reported that a highly active water- and alcohol-soluble pressor substance is formed when renin is incubated with renin activator prepared from blood serum. For this pressor substance we have suggested the name "angiotonin." Angiotonin differs sharply from both renin and activator in that it can be boiled without loss of activity.

Angiotonin is not soluble in organic solvents such as acetone, ether, amyl and butyl alcohol, or chloroform. It appears to be adsorbed readily on norit and metallic sulfides. Regenerated from the crystalline picrate, it retains the fluorescence in ultra-violet light which characterized it before the picrate was formed. The only positive color reaction found is that of Sakaguchi. Additional chemical and physical properties of angiotonin will be presented.

**The Relation of the Excretion of Gastric Juice and of Urine to the Alkalosis of Hydrazine Intoxication in the Rabbit.** BY BYRON M. HENDRIX, D. BAILEY CALVIN, AND MAX M. GREENBERG. *From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston*

It has been shown that hydrazine produces an alkalosis in dogs when administered in suitable doses. Since this substance causes considerable vomiting in dogs, we have used rabbits to study the alkalosis of hydrazine intoxication. We have obtained data on

\* Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 71, 29 (1940).

twenty-four rabbits which show the development of alkalosis in this animal after the administration of hydrazine. A study of the acid content of the stomachs of hydrazine-intoxicated rabbits showed a greater amount of acid than in control animals which had been fasted as long as the experimental ones. The increase in acid in the stomachs was proportionally greater than the increase of available alkali in the plasma. There was not a significant decrease in the chloride of the plasma; hence it may be suggested that some neutral chloride has been mobilized from the extracellular fluid, the base remaining in the blood and the chloride appearing in the stomach as free hydrochloric acid. There was not an increase in urine base or a decrease in urine acid; hence the condition differs from the alkaline tide which follows the normal secretions of hydrochloric acid by the stomach. This effect upon the gastric mucosa occurs so promptly following the administration of hydrazine that it would appear to be a primary effect of the intoxication and not secondary to the action of hydrazine on the liver.

**Cystinuria; the Effect of Feeding Methionine and Other Amino Acids on the Excretion of Cystine.** BY W. C. HESS AND M. X. SULLIVAN. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

We have previously reported\* that feeding both methionine and cystine to a cystinuric did not increase the cystine content of the urine. Recently we have studied another marked case of cystinuria. As previously, estimations were made by the Sullivan method of free cystine in the urine, cystine in the sediment, and cystine liberated by hydrolysis. Feeding 2 gm. of methionine per day for 3 successive days produced an increased output of cystine. The ingestion of 5 gm. of alanine upon 2 successive days produced an equally marked increase in cystine output, while daily feeding of 5 gm. of glutamic acid for 2 days produced no increase in the cystine output. As judged by the total sulfur of the urine, the dietary intake was about the same in each period and increase in water intake had little effect on the total cystine excreted. The possible relationship of the increased cystine out-

\* Hess, W. C., and Sullivan, M. X., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. lv (1938).

put to the specific dynamic action of the amino acids fed is being further studied with other amino acids.

**Exchange of Radiophosphate by Dental Enamel.** BY GEORG VON HEVESY AND W. D. ARMSTRONG. *From the Institute for Theoretical Physics and Laboratory of Zoophysiology, Copenhagen, Denmark*

The enamel of the mature permanent teeth of cats was separated by two independent methods 4 days after subcutaneous injection of very potent preparations of radioactive phosphate. Per gm. the activity of the enamel was 6.7 to 10 per cent of that of dentin. 0.0023 to 0.0076 per cent of the total activity was found per gm. of enamel. The  $P^{32}$  content of the enamel became constant on repeated repurification, indicating insignificant contamination with dentin. Experiments *in vitro* showed that the  $P^{32}$  acquired by enamel *in vivo* is apparently not derived from saliva. The results indicate a slow exchange of phosphorus but not an ability of enamel to undergo significant changes of composition after eruption, as a result of nutritional alterations.

**The Molecular Constitution of the Calcium Phosphates. The Concentration Limits for the Precipitation of Secondary Calcium Phosphate and of Hydroxylapatite.** BY HAROLD CARPENTER HODGE. *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

In electrometric titrations of calcium hydroxide and phosphoric acid at constant temperature, limits have been established for the relative concentrations of these reagents at which (1) only secondary calcium phosphate crystals are precipitated, (2) only hydroxylapatite is precipitated. Intermediate concentrations give mixtures of secondary calcium phosphate and hydroxylapatite. Microscopic examinations of the precipitates were a useful adjunct to x-ray diffraction measurements. The acidity (pH 6 or less) of the solutions from which secondary calcium phosphate crystals are formed makes improbable the hypothesis that  $CaHPO_4$  plays an important initiating rôle in calcification *in vivo*. Furthermore, *in vitro* systems have been prepared in which hydroxylapatite precipitates are shown to be in equilibrium with solutions con-

taining calcium and secondary phosphate ions in analogy with the bone-blood equilibria.

**Anemia from Lysine Deficiency in Deaminized Casein.** BY

ALBERT G. HOGAN, E. L. POWELL, AND RALPH E. GUERRANT.

*From the Department of Agricultural Chemistry, University of Missouri, Columbia*

Rats recover from anemia due to deaminized casein and gain in weight when casein, hydrolyzed casein, or one of the copper salt fractions of hydrolyzed casein is added to the anemia-producing ration. It was concluded therefore that the anemia is caused by a deficiency of, or increased demand for, one or more amino acids. Various amino acids were examined for antianemic activity and eventually it was observed that when anemic rats were supplied with 65 mg. daily of *D*-lysine they recovered and gained in weight. If they received 130 mg., the rate of recovery, and of gain in weight, was accelerated. Various other amino acids were examined but none was effective.

If rats are placed on rations that contain 20 per cent of deaminized casein as the only source of protein, they seldom survive longer than 2 weeks. The addition of 2 per cent of *D*-lysine to such a ration permits slow recovery but no growth, and the addition of 4 per cent permits rapid recovery and a subnormal rate of growth. The explanation for this inordinately large requirement is not now apparent. In tests made up to date the addition of the other essential amino acids had no effect.

**The Isolation of Dihydrotheelin from Human Placenta.** BY

M. N. HUFFMAN, SIDNEY A. THAYER, AND EDWARD A. DOISY.

*From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

The non-ketonic fraction of an extract of 422 kilos of human placenta was obtained from W. W. Westerfeld after his removal of ketones with Girard's reagent. Following the distribution of this non-ketonic fraction between benzene and 0.3 M sodium carbonate (A. Mather, unpublished), bioassay indicated the presence of approximately 16 mg. of dihydrotheelin.

By methods of purification previously used in this laboratory, one-half of the non-ketonic fraction was concentrated to 23 mg.



By naphthoylation 6.80 mg. of pure  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate ( $\approx$  3.08 mg. of  $\alpha$ -dihydrotheelin) were obtained, in spite of the fact that two treatments with norit, two recrystallizations from acetone, and four recrystallizations from alcohol were necessary to obtain a pure product.

This represents 6.16 mg. of  $\alpha$ -dihydrotheelin in the total placental extract as compared with the 12 mg. of pure theelin previously isolated. Bioassay on the ketonic fraction had indicated a total of 15 mg. of theelin present; our bioassays on the non-ketonic fraction have shown 16 mg. of  $\alpha$ -dihydrotheelin and 60 mg. of theelol.

The isolated  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate melted at 191.5–192.5°; a mixed melting point with an authentic sample of  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate (m.p. 194.0–194.5°) was 192.5–193.5°. Analysis, C 82.70, H 6.35; calculated for di- $\alpha$ -naphthoate, C 82.72, H 6.25.

A portion of the  $\alpha$ -dihydrotheelin di- $\alpha$ -naphthoate was hydrolyzed with potassium hydroxide. The phenol was recovered and crystallized from aqueous alcohol after having been treated with a small amount of norit, m.p. 170–170.5°; a mixed melting point with authentic dihydrotheelin (m.p. 172.5–173°) was 173–174°.

**The Cholesterol Content of Plasma and Erythrocytes As Related to Thyroid Activity.** BY HETTIE B. HUGHES. *From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati*

The present report concerns a study of the effects of experimental variations in thyroid activity on the blood cholesterol of the dog. Variations in the free and total cholesterol content of the plasma and erythrocytes of normal dogs have been compared with those produced by (1) administering thyroxine or desiccated thyroid gland to dogs with intact thyroids, (2) thyroidectomy, and (3) administering thyroxine and thyroid gland to thyroidectomized dogs. The results show that none of these procedures altered the free or total cholesterol content of the erythrocytes. The effects on the plasma cholesterol were as follows: (1) Administration of thyroxine or desiccated gland to dogs with intact thyroids had a variable effect on the total cholesterol. Treat-

ment of dogs with high or average normal cholesterol levels reduced the cholesterol to low normal levels. Treatment of dogs with low normal levels was without effect. Moderate doses of thyroid substance were as effective as massive doses. (2) Thyroidectomy was followed by a 100 to 300 per cent increase in plasma cholesterol. (3) Administration of small amounts of thyroxine or desiccated gland to the thyroidectomized dog reduced the cholesterol to normal levels; administration of larger amounts reduced the cholesterol to subnormal levels. (4) During these changes in total cholesterol the proportions of free cholesterol remained constant.

Other experiments have shown that the above changes were not due to fasting, alterations in blood volume, impaired or accelerated excretion of biliary cholesterol, or differences in retention of cholesterol by tissues.

**A Study of the Effect of Altitude on Basal Metabolism.** BY ALBERTA ILIFF, ANNA MARIE DUVAL, GLADYS M. KINSMAN, AND ROBERT C. LEWIS. *From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver, and the School of Home Economics, Oklahoma Agricultural and Mechanical College, Stillwater*

In a study of the energy metabolism of the children of the Child Research Council at Denver during the past 8 years, sufficient data have been accumulated to establish normals for the different age groups of children from 2 through 15 years old, inclusive. Reports in the literature seem to indicate that, at least for elevations under 10,000 feet above sea level, altitude is not a factor in the determination of basal metabolism. However, the question has been raised so often as to whether the normal values established in Denver (altitude 5280 feet) can be used as standards at lower altitudes that it seemed desirable to subject this question to experimental test. Since it was impracticable to make such a study on children, adults (five women and two men) were used. The basal metabolism of all seven subjects was determined at Denver, Colorado (altitude 5280 feet), and at Stillwater, Oklahoma (altitude 910 feet), and in case of four of the subjects also at Eldora, Colorado (altitude 8720 feet). The subjects remained at each altitude sufficiently long to become acclimatized as

judged by certain values (erythrocyte count, hemoglobin, red cell volume, and blood specific gravity) for the measurement of physiological function. Basal metabolism determinations were made almost daily during the period of adjustment to the change in altitude and for some days after acclimatization had become accomplished. The results of this study indicate that altitude up to 8720 feet has no influence on the determination of basal metabolism.

**Chlorophyll As a Part of the Photosynthetic Mechanism.** BY  
O. L. INMAN. *From the C. F. Kettering Foundation for the Study of Chlorophyll and Photosynthesis, Antioch College, Yellow Springs, Ohio*

By the use of proteolytic and lipolytic enzymes acting on *Trifolium repens* leaf triturates made by grinding the leaves in water or buffer with sand and filtering out the cells, it has been shown that the stability of chlorophyll to carbon dioxide and copper is decreased. So long as the protein or lipids of the triturate remain undisturbed chlorophyll is very stable. Finely divided metallic copper added to a triturate from pH 6.8 to 7.0 will slowly replace the magnesium in the chlorophyll molecule but the addition of 5 mg. of trypsin or steapsin to 5 cc. of triturate causes a rapid increase in the rate of this reaction. Bubbling pure carbon dioxide through a triturate at pH 6.8 to 7.0 for 3 hours will not cause the chlorophyll to lose its magnesium but the addition of a few mg. of trypsin or steapsin will bring about the formation of pheophytin in 30 minutes without a further lowering of the pH. Pepsin, papain, and amylopsin under the same conditions show no accelerating action. Detergents such as Duponol PC bring about the loss of magnesium from the chlorophyll molecule even at pH 8.00 and dissolve the chlorophyll from its substrate, causing the loss of magnesium from the chlorophyll molecule. According to Anson such detergents denature proteins. A 4 per cent ethyl alcoholic colloidal solution of pure chlorophyll *a + b* treated with Duponol PC undergoes the same change but at a much slower rate. Such evidence indicates that the proteins and lipids are closely associated with chlorophyll which is a part of the photosynthetic mechanism.

**Hormones of the Digestive Tract.** BY A. C. IVY. *From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

Three gastrointestinal hormones have been sufficiently studied and the results confirmed to warrant the conclusion that their existence is established. These are secretin, cholecystokinin, and enterogastrone. *Secretin* has apparently been crystallized but its structure has not been determined. It can be and has been used in man to examine pancreatic secretion, as histamine is used to test gastric secretion. It also specifically stimulates choleresis. *Cholecystokinin* has been concentrated sufficiently to show that it causes the human gallbladder to contract and evacuate. Chemically it is closely related to, but not identical with secretin. *Enterogastrone* is produced by the intestinal mucosa and is responsible, in part at least, for the depression of gastric secretion and motility when considerable quantities of fats or sugar are ingested. A substance recently termed *urogastrone* has been found in canine and human urine. When injected into man or dog, it inhibits the gastric secretory response to a meal or histamine. This substance disappears from canine urine after pyloroenterectomy, which indicates, but does not prove, that the substance is made by the intestinal mucosa. Regarding *gastrin*, it is established that a humoral mechanism is in part responsible for postcibal gastric secretion. Whether histamine or some other substance is the humoral agent remains to be determined. Claims for the existence of a non-histamine gastrin have not been confirmed. The evidence indicates that a hormone, *enterocrinin*, exists which stimulates the secretion of intestinal juice (Nasset). We have failed to confirm the existence of *incretin* or *duodenin*, which is supposed to stimulate insulin production. The existence of *enterocin* (motor hormone) and *villikin* as specific entities is problematic.

**The Relation between the Interstitial Cell-Stimulating and Thyrotropic Effects of the Anterior Pituitary.** BY H. JENSEN AND SIBYLLE TOLKSDORF. *From the Biochemistry Laboratory, The Squibb Institute for Medical Research, New Brunswick*

We observed that the thyrotropic effect of various anterior

pituitary preparations was always associated with the interstitial cell-stimulating principle. It has been shown recently that the interstitial cell-stimulating hormone is identical with the luteinizing principle. In order to determine whether these two physiological responses may perhaps be due to only one principle we attempted to fractionate the interstitial cell-stimulating and thyrotropic activities by various chemical manipulations. The interstitial cell-stimulating activity was determined in hypophysectomized, immature female rats by intraperitoneal administration. The thyrotropic effect was determined in day-old chicks by the method of Smelser. In both cases, the minimal stimulation was determined histologically. We found that the minimal effective dose for repair of the ovarian interstitial tissue in hypophysectomized rats corresponded closely to that for minimal thyrotropic effect in the chick. The various preparations studied biologically for both responses always gave approximately the same ratio. So far we have not been able to separate, by chemical means, the two physiological effects. The various chemical methods employed by us will be discussed.

Such interstitial cell-stimulating agents as chorionic gonadotropin and pregnant mare serum were found to be devoid of thyrotropic potency. The results of our comparative physiological studies lead us to believe that the two effects, *i.e.* stimulation of the interstitial tissue and of the thyroid, are produced by one hypophyseal principle, the effects depending on the dose level and on the route of administration.

**Some Observations on the Behavior of the Sulfinic and Sulfonic Acids Corresponding to Cysteine.** BY BEATRICE KASSELL.  
*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

Recent evidence indicates that cystine may hydrolyze, forming cysteine and acidic derivatives. The sulfinic and sulfonic acids corresponding to cysteine were studied, particularly under the conditions of protein hydrolysis.

Neither acid is oxidized by phospho-18-tungstic acid (pH 5.7) or by porphyrindin (0°, pH 7.2). Both acids are resistant to Zn-HCl reduction at 25°; at 70° the sulfinic acid is partially reduced to cysteine (20 per cent in 1 hour).

Digestion with 57 per cent HI (150°, 5 hours) converts the sulfinic acid quantitatively to cysteine, while cysteic acid is relatively stable (10 per cent reduction). The sulfinic acid is reduced to cystine by 2 N HI at 25° or by heating in 6 N HCl with cysteine, artificial humin (made by heating mannose with tryptophane and tyrosine), or with protein hydrolysates containing cysteine or humin.

Digestion of the sulfinic acid with 6 N HCl at 130° results in partial decomposition, but does not yield products which react with phosphotungstic acid in the presence or absence of sulfite. After treatment of evaporated HCl digests with 57 per cent HI, 70 to 80 per cent of the sulfinic acid is recovered as cysteine and about 10 per cent as H<sub>2</sub>S.

The cystine plus cysteine content of certain proteins, *e.g.* egg albumin, is lower in HCl than in HI hydrolysates, while HI digestion of the former yields the same values as direct HI digestion. The observations on the behavior of the sulfinic acid corresponding to cysteine suggest the possibility that this substance is responsible for the discrepancy.

**The Adrenal Cortex.** BY EDWARD C. KENDALL. *From the Department of Biochemistry, The Mayo Foundation, Rochester, Minnesota*

From the adrenal cortex five crystalline steroids have been separated: A dehydrocorticosterone, B corticosterone, E C<sub>17</sub>-hydroxy A, F C<sub>17</sub>-hydroxy B, desoxy B, and an amorphous fraction, all of which will maintain life in adrenalectomized animals. No one compound can be described as the life-maintaining hormone. For maintenance of adrenalectomized dogs the amorphous fraction is the most active, followed by desoxy B acetate, desoxy B, A and B, E and F. Other physiological effects indicate sharply defined qualitative differences among these compounds. In rats desoxy B and its acetate cause increase, B and E acetates a marked loss in body weight; the amorphous fraction has little effect. Sodium is increased in serum most by desoxy B and its acetate, less by E acetate; A, B, and the amorphous fraction have much less effect. Potassium is decreased in serum most by desoxy B acetate; desoxy B, A, B, and the amorphous fraction do not decrease potassium in serum below normal.

Carbohydrate metabolism is affected most by A and B, less by E, desoxy B has little effect, and the amorphous fraction still less. A and B produce atrophy of adrenal and thymus glands in normal male rats. Acetates of B and E cause almost complete atrophy of the thymus gland. Desoxy B and its acetate and the amorphous fraction do not cause significant atrophy. Ingle has shown wide variations in the effect of each of these compounds on the capacity of muscles for work; A, B, and E are all more active than desoxy B. These results show the changes in physiological response produced by hydroxyl and ketone derivatives of desoxycorticosterone. Formation of an ester on the alcohol group of C<sub>21</sub> produces both qualitative and quantitative effects.

**A Rapid Method for the Separation of Serum Albumin and Globulin.** BY GEORGE R. KINGSLEY. *From the Division of Biochemistry, Philadelphia General Hospital, Philadelphia*

If 4 cc. of ether are immediately added after globulin is precipitated from 1 cc. of serum with sodium sulfate, as in the Howe method, and the mixture vigorously shaken for 20 to 30 seconds and then centrifuged in a test-tube (17 to 20 mm.) for 5 minutes at about 2200 R.P.M., the globulin separates in a compact layer between the sodium sulfate solution and the ether. By tilting the tube the globulin layer is easily disengaged from the sides and samples can be withdrawn by pipette for albumin analysis.

Globulin separation by this method is complete, since the albumin nitrogen values of the centrifugates are in good agreement with those of filtrates obtained by proved technique. The protein was determined by the biuret method of the author\* and by the Kjeldahl method.

When the globulin from 1 cc. of serum is extracted with ether and dissolved in 1 cc. of 3 per cent sodium chloride, evacuated until no odor of ether remains, then mixed with 15 cc. of 23 per cent sodium sulfate and centrifuged, no separation takes place. However, addition of ether again permits a sharp separation. When a heavier solvent such as carbon tetrachloride is used, the globulin separates between the two liquid phases beneath the sodium sulfate solution. Therefore the globulin or globulin-

\* Kingsley, G. R., *J. Biol. Chem.*, **131**, 197 (1939).

lipid complex appears to absorb enough of the lipid solvent to change its density sufficiently for separation by centrifugation. These observations provide the basis for a rapid and highly practical method for separation of globulin from albumin.

### **The Effect of Change of Altitude on the Blood of Human Subjects.**

BY GLADYS M. KINSMAN, ANNA MARIE DUVAL, ALBERTA ILIFF, AND ROBERT C. LEWIS. *From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver, and the School of Home Economics, Oklahoma Agricultural and Mechanical College, Stillwater*

In conjunction with studies made to observe the influence of altitude on the basal metabolism, blood studies which included erythrocyte counts, hemoglobin determinations, red cell volumes, and blood specific gravities were conducted primarily as a means of determining physiological adjustment to the changes in altitude. These determinations were made on the same subjects, five women and two men, at two different altitudes, 910 feet (Stillwater, Oklahoma) and 5280 feet (Denver, Colorado) above sea level. With four of the subjects, three women and one man, the studies were extended to a third altitude (8720 feet above sea level at Eldora, Colorado). From the values obtained for the above measurements, the mean corpuscular hemoglobin, the mean corpuscular volume, and the mean corpuscular hemoglobin concentration were calculated. Although physiological changes in the blood are known to occur with altitude, this conclusion has been drawn largely from studies made on different groups of individuals at different altitudes. Not often is opportunity afforded, as was the case in this study, to observe these effects of altitude on the same individuals by identical techniques performed by the same workers. Increases in hemoglobin content, erythrocyte count, and red cell volume occurred in all subjects with increases in altitude, but there was considerable variation in the increments observed and in the rate of adjustment to the changes in altitude.

**The Determination of Blood Pyruvate in the Presence of Acetate.** BY DANIEL KLEIN. *From the Metabolic Laboratory, Philadelphia General Hospital, Philadelphia*



The method of Peters and Thompson\* for the determination of pyruvate could not be applied unmodified to diabetic blood, because of the incomplete decomposition of the dinitrophenylhydrazones of acetoacetic acid. The same applied to the more recent micromethod of Lu when tried out on a macro scale.

A modified procedure has been developed to avoid this source of error. The method differs from that of Peters and Thompson in four details. Tungstic acid replaces trichloroacetic acid. Acetoacetic acid dinitrophenylhydrazone is destroyed by allowing the first ethyl acetate extract to stand overnight. Extraction of pyruvic acid dinitrophenylhydrazone is made with  $m/15$  disodium phosphate only. 0.5 N aqueous sodium hydroxide replaces alcoholic potassium hydroxide used to develop the color for photoelectric photometry.

Pyruvic acid in 1 per cent tungstic acid (Folin-Wu) solutions was recovered to the extent of 90 to 100 per cent. Addition of pyruvate to blood resulted in recoveries of 75 to 80 per cent. Acetoacetic acid in amounts ranging up to 250 mg. per cent gave readings not exceeding 0.2 mg. per cent as pyruvic acid.

Preliminary studies of diabetes mellitus in different stages of control, including diabetic coma, indicate that the blood pyruvate level is normal, unless there is associated a severe vitamin B<sub>1</sub> deficiency.

**Intravenous Galactose Tolerance.** BY ALFRED E. KOEHLER AND ELSIE HILL. *From the Sansum Clinic and the Santa Barbara Cottage Hospital, Santa Barbara*

The oral galactose tolerance test for liver function frequently gives results that are not consistent with clinical or postmortem findings. That some of these discrepancies are due to variation in galactose absorption has been shown by giving the galactose intravenously at a constant rate. 50 gm. of galactose were given as a 50 per cent solution over a period of 2 hours.

In a normal person the blood galactose reaches a maximum of about 35 mg. above the fasting level (non-fermentable reducing substances) in approximately 1½ hours after the start and then drops appreciably before the end of injection. Half an hour

\* Peters, R. A., and Thompson, R. H. S., *Biochem. J.*, **28**, 916 (1934).

after end of injection the galactose value has usually returned to the fasting value and may subsequently drop below.

In certain types of liver disorders the blood galactose rises markedly during injection and there usually is a continued rise until injection is stopped. This failure on the part of the body to stimulate the rate of removal of galactose from the blood stream, as happens normally, is in itself a criterion of liver damage.

Certain subjects have shown an abnormally poor intravenous galactose tolerance in which liver disease was not suspected, nor could such liver damage later be proved, although it could, of course, have existed.

Concomitant with the rise of blood galactose after injection there is a rise in the blood glucose (fermentable fraction). In the mildly diabetic patient this rise is quite appreciable and becomes a good index of the glucose tolerance.

#### **The Intravenous Injection of Acetone in Normal and Diabetic Subjects.** BY ALFRED E. KOEHLER AND EMANUEL WINDSOR.

*From the Sansum Clinic, the Santa Barbara Cottage Hospital, and the Santa Barbara General Hospital, Santa Barbara*

The fate of acetone (10 gm. in 200 cc. of saline) injected intravenously for 2 hours at constant rate was studied in eighteen normal and twelve diabetic subjects. There were no symptomatic effects except varying degrees of drowsiness toward the end of the injection.

In the non-diabetic group blood acetone plus acetoacetic acid rose from  $0.8 \pm 0.2$  mg. per 100 cc. to  $19.6 \pm 1.3$  mg. at the end of injection and still was  $18.1 \pm 1.3$  mg. 4 hours after the end of injection. In the diabetic group the values were similar. In neither group were blood  $\beta$ -hydroxybutyric acid or sugar levels appreciably altered.

In the non-diabetic group acetoacetic acid rose from  $0.44 \pm 0.19$  mg. per 100 cc. to  $6.9 \pm 0.7$  mg. at end of injection and was  $5.2 \pm 0.7$  mg. 4 hours later.

In the normal group simultaneous injection of glucose (100 gm.) or insulin (30 units) with the acetone caused no appreciable change in acetone or acetoacetic acid in either blood or urine as compared with the subjects receiving acetone alone. The urinary excretion paralleled the blood ketone values.

Considering that there is some loss of acetone through the lungs and kidneys, the prolonged high acetone level after injection is evidence that diabetic and normal persons convert acetone very slowly if at all. The acetoacetic acid rise does, however, indicate that injection of acetone brings about a disturbance of ketone metabolism.

**A Rapid Photoelectric Method for the Determination of Vitamin A and Carotene in Milk.** BY C. J. KOEHN. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

A rapid method has been developed for the photoelectric determination of vitamin A and carotene in milk. The unsaponifiable fraction was extracted with ether after digestion of the milk with alcoholic KOH on a water bath for 10 minutes. This fraction was purified and transferred to chloroform. The carotene and vitamin A were determined by means of a photoelectric colorimeter, the carotene by measurement of the light absorption at  $440\text{ m}\mu$  and vitamin A by measurement of the light absorption at  $620\text{ m}\mu$  by the blue compound found in the Carr-Price reaction.

The vitamin A value of the milk was also determined by biological assay with rats and a factor was derived for converting colorimetric readings into international units. After correction for the carotene and xanthophyll present in milk, it was found that the factor for converting colorimetric readings into international units of vitamin A was appreciably higher for milk than for cod liver oil.

**Creatine-Creatinine Metabolism and the Hormones. V. Effect of Parenteral Injection of Epinephrine, Eschatin, Percorten, and Saline into Normal and Adrenalectomized Rats upon Muscle Creatine and Creatine-Creatinine Excretion.** BY A. LINK KOVEN, PHILIP PIZZOLATO, AND HOWARD H. BEARD. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

The injection of 0.1 cc. of 1:1000 epinephrine into normal and adrenalectomized rats resulted in the excretion of urines that gave a fairly intense Jaffe reaction with alkaline picrate after they were hydrolyzed with HCl. All of these urines tested, however,

reduced Benedict's qualitative sugar solution and, after incubation with the creatinine enzyme of Miller and Dubos, the color of the Jaffe reaction after addition of alkaline picrate was *not* destroyed. The result shows that epinephrine does not give a true creatinuria under the above conditions.

The urine samples, after the injection of 1 cc. of eschatin or 0.5 cc. of percorten (desoxycorticosterone acetate in sesame oil), did not reduce Benedict's qualitative sugar reagent and the color of the Jaffe reaction was destroyed after the samples were incubated with the creatinine enzyme. This indicates that these hormones cause a true creatinuria. These hormones themselves give the Jaffe reaction with alkaline picrate and reduce Benedict's solution. They were not, however, excreted as such in the urine (negative Benedict's test). The injection of 18 or 50 mg. of physiological saline into the adrenalectomized animals gave about the same creatinuria as these cortical hormones. It is, therefore, possible that they produce creatinuria owing to their water- and salt-retaining effects.

Adrenalectomy alone produced some slight creatinuria which was due to the anesthetic and trauma of the operation. Injection of the above hormones did not affect the concentration of muscle creatine in any of the animals studied.

**The Nephelometric Determination of Quinine in Small Amounts by Means of the Photoelectric Colorimeter.** BY GRANVIL C. KYKER, BAILEY D. WEBB, AND JAMES C. ANDREWS. *From the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

The studies previously reported on the determination of small amounts of quinine in blood have been continued by studying the suitability of the Evelyn photoelectric colorimeter for use in the modified Vedder and Masen nephelometric method. Curves of galvanometer readings *versus* concentrations of pure quinine throughout the biological range have been determined and it has been shown that a very satisfactory slope results when the readings are made with a blue filter (400  $\mu$ ) on concentrations of about 1 to 10 mg. per liter. Determinations on unknown quinine samples can be made with much greater accuracy and less eye fatigue than when a visual method is used.

Using the photoelectric colorimeter, we have rechecked and

confirmed the curve of acid concentration *versus* completeness of precipitation of the quinine silicotungstate and still find 0.03 M HCl to be the optimal concentration. An elaborate series of studies has also been made of the effect of allowing various periods of time to elapse between precipitating the quinine silicotungstate and taking the galvanometer readings. The effect of agglutination of the precipitated particles has been found to be much less pronounced in 0.03 M than in the 0.5 M HCl recommended by Vedder and Masen. The method is now being extended to the determination of quinine obtained from ether extracts of blood.

**The Reaction of Sulfur Compounds with Nitrous Acid.** By THEODORE F. LAVINE AND WILLIAM M. WHITELEY. *From the Lankenau Hospital Research Institute, Philadelphia*

Cysteine (R—SH) and cystine (R—SS—R) have long been known to yield high results in the Van Slyke procedure for the estimation of amino nitrogen, while correct results are obtained with cysteic acid (R—SO<sub>3</sub>H). It has been found that cystine disulfoxide (R—(SO)<sub>2</sub>—R) and cysteine sulfinic acid (R—SO<sub>2</sub>H) yield results similar to cysteine and cystine. Theoretical values are obtained in all cases when a KI-acetic acid mixture is substituted for the usual acetic acid. The determinations were carried out in the volumetric apparatus.

The production of "extra nitrogen" by the sulfinic acid and the concomitant formation of sulfate suggest cleavage of sulfur from the organic molecule with an inorganic sulfur compound thus being responsible for the "extra nitrogen." Such a cleavage might result in the formation of sulfoxylic acid (S(OH)<sub>2</sub>) or thiosulfuric acid (H<sub>2</sub>S<sub>2</sub>O<sub>3</sub>). Both of these compounds (sodium formaldehyde sulfoxylate and sodium thiosulfate) liberate nitrogen in the Van Slyke procedure but only the thiosulfate values are reduced effectively by KI-acetic acid. Of other inorganic sulfur compounds, sulfite and hyposulfite yielded appreciable amounts of nitrogen, sulfide a small amount, and dithionate and tetrathionate negligible amounts.

It is assumed that the nitrogen-producing reaction is not an oxidation, since nitrous acid is usually reduced to oxides of nitrogen, but rather a rearrangement of a nitrogen-sulfur compound

to a sulfonamide structure similar to sulfamic acid,  $\text{HO}-\text{SO}_2-\text{NH}_2$ , which reacts normally with nitrous acid to yield nitrogen.

**The Percutaneous Administration of Estrogens Followed by Progestin in Inducing Sexual Receptivity in Spayed Guinea Pigs.** BY JOHN A. LEIGHTY, H. J. WICK, AND B. E. JEFFRIES.  
*From the Lilly Research Laboratories, Indianapolis*

Sexual receptivity was induced in the spayed guinea pig by estrone plus progestin, and by stilbestrol plus progestin, when the substances were applied percutaneously. Estrone was about twice as effective in this respect as stilbestrol.

The estrogens were administered in oil, while progestin was dissolved in an alcohol-glycerol solution in some experiments and in oil in others with only a slight difference in results.

Under the conditions of the experiments, when the amount of estrogen rubbed on was held constant, more progestin was required percutaneously than by injection to obtain comparable effects.

These results are of interest because they suggest the possibility of the use of the percutaneous method of administration of progestin in clinical cases, thus avoiding the present rather unsatisfactory use of injections of oils.

**Fractionation of Serum Non-Protein Sulfur.** BY T. V. LETONOFF AND JOHN G. REINHOLD. *From the Division of Biochemistry, Philadelphia General Hospital, and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Total sulfur together with inorganic, ethereal, and neutral sulfur has been determined in protein-free filtrates of serum prepared by means of uranium acetate. Nine normal individuals showed in serum the following concentrations: total non-protein sulfur, average 2.84 (range 2.40 to 3.10), inorganic sulfur, average 1.09 (1.00 to 1.22), ethereal sulfur, average 0.80 (0.4 to 1.19), and neutral sulfur, average 0.94 (0.57 to 1.48) as mg. per cent. Similar values were found for citrated plasma. Inorganic sulfate added to citrated plasma is recovered quantitatively in the total and inorganic sulfate fractions. Analyses of protein-free filtrates of serum prepared by addition of 1 cc. of 20 per cent trichloroacetic

acid solution per cc. of serum gave values significantly higher for total, inorganic, and neutral sulfur, while ethereal sulfur usually was lower. Doubling the concentration of trichloroacetic acid increased the differences, but additional trichloroacetic acid had no further effect. Analyses of dialysates of serum agreed well with figures for inorganic and ethereal sulfate found in uranium acetate filtrates. Total sulfur and neutral sulfur were higher in the dialysates, although not as high as in trichloroacetic acid filtrates. Addition of uranium acetate to the dialysates gave values equal to those found in uranium acetate filtrates. This may have been due to undetected traces of protein. The results support the belief that trichloroacetic acid in high concentrations attacks certain sulfur-containing compounds of serum and alters the distribution of sulfur in the fractions studied.

**The Quantitative Precipitation of Gonadotropin from Normal Urine.** BY LOUIS LEVIN. *From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York*

The recovery of gonadotropin from specimens of normal male urine by tannic acid precipitation and alcohol precipitation has been compared. The extract of the initial alcohol precipitate is too toxic to permit assay of the small amounts of gonadotropin present (less than 30 mouse uterus units per 24 hour specimen). The alcohol extracts were consequently partially detoxified by dialysis, necessitating reconcentration (accomplished by acetone precipitation or by evaporation in a stream of air) of the dialyzed solutions. The activity of the dialyzed, reconcentrated extracts of the alcohol precipitates was uniformly lower (by as much as 50 per cent) than that of the corresponding tannic acid preparations.

Investigation of the low yields obtained by the alcohol precipitation shows that 10 to 15 per cent of the activity remains in the insoluble residue of the alcohol precipitate. A further fraction (25 to 30 per cent) may be lost during the manipulations incident to dialysis and as much as 20 per cent is lost during the air evaporation.

These findings demonstrate (1) the inadvisability of avoidable manipulation when dealing with gonadotropin in amounts as small as those of normal urines, (2) that the original claims for

the quantitative precipitation of gonadotropin by tannic acid were not unjustified, and (3) that tannic acid is the most effective precipitant yet proposed for quantitative recovery of urinary gonadotropin of normal individuals.

Urinary gonadotropin excretion curves for one normal and one pre-pregnant menstrual cycle will be shown.

**The Ascorbic Acid Content of the Blood of the Eskimo.** By VICTOR E. LEVINE. *From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha.*

In the course of a health survey conducted at Point Barrow under the auspices of the United States Public Health Service, we determined towards the end of January, 1938, the ascorbic acid content of the blood of thirty Eskimo children. The range for six children was 0.15 to 0.20 mg. per 100 cc.; for seven, 0.21 to 0.25 mg.; for eight, 0.26 to 0.30 mg.; for seven, 0.31 to 0.40 mg.; and for two, 0.45 to 0.55 mg. Capillary fragility varied from 7 to 17 cm. of Hg. Utilizing Dalldorf's standards,\* we found twenty-eight children had subacute scurvy at the time of the examination. Orange juice raised ascorbic acid values and capillary resistance, though not always in the same ratio.

The diet of the Point Barrow Eskimo by the end of January was devoid of fresh meat. Only reindeer killed several months before was available. Berries are not preserved in seal oil for winter use as in other villages.

Physical examination revealed acute, subacute, or chronic hypertrophic rhinitis, the nasal mucous membranes being edematous and the capillaries congested. Bleeding occurred frequently, even at such slight provocation as the insertion of the nasal speculum. Epistaxis, now common in winter and early spring and disappearing with the advent of fresh meat, we regard as diagnostic of scurvy among Eskimos. Høygaard† in Greenland has made a similar suggestion. Our tuberculin tests gave markedly severe reactions. Birkhaug‡ has demonstrated in vitamin C deficiency increased severity of the cutaneous response to tuberculin.

\* Dalldorf, G., *Am. J. Dis. Child.*, **46**, 794 (1933); *Bull. New York Acad. Med.*, **15**, 544 (1939).

† Høygaard, A., *Nord. Med. Tidsskr.*, **16**, 1647 (1938).

‡ Birkhaug, K. M., *Acta tuberc. Scand.*, **13**, 45 (1939).



**The Effect of the Thyrotropic Hormone on the Composition and Metabolism of the Thyroid Gland.** BY MILAN A. LOGAN, J. E. VANDERLAAN, AND W. P. VANDERLAAN. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

Thyrotropic hormone of the pituitary was administered to guinea pigs daily for 6 days. In the first 24 hours following the administration of the hormone, the water, chloride, and total phosphorus content per gm. of thyroid tissue increased markedly. At the same time, the oxygen consumption of the thyroid tissue, measured *in vitro* by the Warburg technique, increased more than 60 per cent. The increases persisted for 2 days, following which the oxygen consumption and the determined constituents of the tissue approached normal values. Accompanying these changes was an increase in the size of the glands. The results are compared with the histological changes observed.

**Purine Content of Human Cardiac and Voluntary Muscle.** BY GEORGE H. MANGUN AND VICTOR C. MYERS. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

Studies on human heart muscle have shown that creatine, phosphorus, and potassium are decreased in myocardial failure. The relative losses of creatine and phosphorus indicated that the loss of phosphorus could not be entirely accounted for as being due to a breakdown of phosphocreatine alone. Studies on the dog heart in late aortic insufficiency indicated that adenylypyrophosphate may be lost to an even greater extent than phosphocreatine. Since adenylypyrophosphate breaks down following death and therefore cannot be determined in autopsy tissue, a method was developed to determine the extractive purine nitrogen after deamination of the aminopurines with nitrous acid. In nine cases, the total extractive purine N before deamination averaged 38.1, 21.2, and 32.7 mg. per 100 gm. in the left and right ventricles and pectoralis major respectively. After deamination with nitrous acid, the oxypurine N averaged 34.4, 18.9, and 29.1 mg. per 100 gm. in the left and right ventricles and pectoralis major respectively. The lowest values encountered in the left ventricle to date were in cases with myocardial insufficiency. Total extractive purine N and oxypurine N values were decreased per unit of fresh tissue and per unit of total solids. Experiments

performed so far indicate that purines, as in the case of creatine, phosphorus, and potassium, are lost from the failing heart and may possibly become an important factor in myocardial insufficiency.

**The Metabolism of Renal Cortical Tissue in Experimental Hypertension.** BY MORTON F. MASON, ALFRED BLALOCK, AND C. S. ROBINSON. *From the Departments of Biochemistry and Surgery, Vanderbilt University School of Medicine, Nashville*

The  $Q_{O_2}$ , R.Q., and aerobic glycolysis of slices of renal cortical tissue from normal dogs and dogs with experimental hypertension produced by partial constriction of the renal artery were compared. The gas exchange was determined by the second method of Dickens and Simer, in bicarbonate, glucose-Ringer buffer. The  $Q_{O_2}$  values of renal cortex slices from nine normal dogs averaged 14.0; in the case of seven hypertensive dogs in which the elevation of blood pressure had been present for from 5 weeks to 7 months, the  $Q_{O_2}$  averaged 14.3. The spread of values was not materially different in either series of animals. The same may be said of the respiratory quotients, which averaged 0.86 in both groups of dogs. No differences in aerobic glycolysis were observed. Preliminary observations suggest a reduction in ammonia formation in the case of "hypertensive" cortical tissue.

**A Method for the Separation and Determination of Urinary Estrogens.** BY ALAN MATHER. *From the Research Laboratories of the Worcester State Hospital, Worcester*

On the basis of the distribution ratios of pure estrogens for various solvent pairs, and from quantitative studies on the several steps used, the efficiency of the separation of the estrogens in urine has been determined, with the Marrian spayed mouse procedure for assay. Bioassay is necessary for estimating the estrogen content of male urines but the method of separation should be applicable to the colorimeter determination used for pregnancy urines.

Optimal conditions for hydrolysis are approximated by heating in normal hydrochloric acid at 100°; 15 minutes suffice for male urines; 1 hour is required for maximum values with pregnancy urines. The rates of destruction of the pure compounds under these conditions range from 20 to 40 per cent per hour.

Ethyl ether has been found to be the most suitable initial

extractant from the neutralized urine (pH 9). The separation of estriol from estrone and estradiol is effected by partition between 0.3 M sodium carbonate and benzene. The benzene to alkali ratios are as follows: estriol 1:40, estrone 250:1, and estradiol 40:1. Extraction of a carbonate eluate of the above ether residue with benzene, followed by cross-extraction of each fraction, gives a separation of well over 95 per cent. The determination of estradiol in the benzene fraction has been satisfactorily accomplished by means of the ketonic reagent, carboxy methoxyl amine, as described by Huffman and Doisy. The estrone is calculated by difference.

The method has been applied to urines of normal and schizophrenic male subjects before and after injections of testosterone propionate.

**Thyroid Hormone in Tissues.** BY J. F. McCLENDON, WILLIAM C. FOSTER, AND W. G. KIRKLAND. *From the Hahnemann Medical College and Hospital, Philadelphia*

5 gm. of tissue are frozen in liquid nitrogen, pulverized, and extracted 1 hour in a shaking machine with 100 cc. of methanol; then extracted a second time with 100 cc. of acetone in a 3 foot glass extractor, with a 9 inch cellulose (Visking) extension. The residue is burned in a platinum tube and analyzed for iodine by the McClendon-Bratton method. This is called thyroid hormone iodine because 333 determinations on blood show a 0.627 correlation with basal metabolic rate. Analysis of fresh steer muscle gave a value of 6  $\gamma$  per 100 gm., compared to 24  $\gamma$  for the mouse, which has a higher basal metabolic rate per kilo. Feeding a rat 20 grains of thyroid raised the thyroid hormone in muscle from 9 to 11  $\gamma$ , and liver from 8 to 58  $\gamma$ .

Average values for the thyroid hormone iodine in human tissue were as follows: muscle 6, heart 6, liver 8, struma ovarii 486, metastasis of the same in the ischium 323. The thyroid hormone in struma ovarii and in metastasis of the same in bone is probably formed in these tissues, which resemble thyroid histologically.

**Effects of Different Levels of Protein in the Diet of the Rat.** BY RICHARD H. MCCOY. *From the Biochemistry Department, The Wistar Institute of Anatomy and Biology, Philadelphia*

The effects of different levels of protein in the diet of the rat when the concentration of vitamins, fats, and inorganic salts remains constant have been studied. A part of the dextrin of the basal diet was replaced with highly purified vitamin-free, salt-free casein to produce diets containing 15, 25, and 40 per cent protein.

In the paired feeding method the 40 per cent diet produces the most rapid growth, the highest percentage of nitrogen, but the lowest percentage of fat of the empty carcass weight. The 15 per cent diet produces the slowest growth, the lowest percentage of nitrogen, but the highest percentage of fat. In the *ad libitum* feeding method only small differences in growth rate are observed between the 25 and 40 per cent diets, yet both are distinctly superior to the 15 per cent diet. The percentage of fat in animals on the 40 per cent diet is consistently lower than in those from either the 25 or 15 per cent diets.

To date the reproductive performance of rats on these diets is as follows: 15 per cent, 227 litters totaling 1626 young of which 60 per cent have been successfully weaned; 25 per cent, 239 litters, 1816 young, 77 per cent weaned; 40 per cent, 283 litters, 2274 young, 77 per cent weaned. The average weaning weight is highest on the 25 per cent and lowest on the 15 per cent diet.

**Synthesis of Members of the Vitamin B Complex in the Rumen of the Cow.** BY L. W. McELROY AND HAROLD GOSS. *From the Division of Animal Husbandry, University of California, Davis*

We have recently reported\* that considerable amounts of thiamine, riboflavin, pantothenic acid, and vitamin B<sub>6</sub> were found in the rumen contents of sheep fed a ration deficient in these vitamins. This ration has now been fed to a cow with a rumen fistula for 4½ months. After 80 days on the experimental diet the cow gave birth to a normal calf. From birth to 43 days of age the calf fed solely on its dam's milk and remained in excellent condition, gaining an average of 1.7 pounds per day.

Samples of both the dried rumen contents and the milk of the cow are being tested for vitamins of the B complex. The results of one chick assay indicate that the dried rumen contents contain

\* McElroy, L. W., and Goss, H., *J. Biol. Chem.*, **130**, 437 (1939).

slightly in excess of 70 micrograms of pantothenic acid per gm. and the dried whole milk about 18 micrograms.

By the fluorescence test of Whitnah the milk contains  $1.4 \pm 0.15$  micrograms of riboflavin per gm., while the milk of twelve cows on a normal ration in the University herd averaged  $1.5 \pm 0.25$  micrograms per gm.

Bandier's colorimetric method for nicotinic acid was applied to the rumen contents and indicated the presence of about 150 micrograms of nicotinic acid per gm. of dried material. The test gave negative results when applied to the diet. Work is in progress to test the curative value of the rumen contents for blacktongue in dogs.

**Observations on Fat Synthesis and Metabolism.** BY E. W. MCHENRY AND GERTRUDE GAVIN. *From the School of Hygiene, University of Toronto, Toronto, Canada*

It has previously been reported that thiamine causes fat synthesis in rats and in pigeons. When this effect of thiamine is studied with a choline-free diet, fatty livers are produced but the amount of liver fat is quickly restored to normal by small doses of choline. In rats given an alcohol-soluble fraction of beef liver, fat synthesis is greatly augmented and fatty livers, different from those caused by thiamine, are produced. These fatty livers are highly resistant to the lipotropic action of choline but readily respond to lipocaic. A procedure for the rapid assay of lipocaic is thus available. The administration of the liver fraction causes a marked increase in liver cholesterol, although no cholesterol is furnished by the basal diet or supplements. Lipocaic diminishes the amount of liver cholesterol. It is suggested that lipocaic affects the transport of cholesterol, while choline is mainly concerned with the transport of neutral fat. Substances such as rice polish concentrate and yeast have an effect similar to that of lipocaic. The crude liver fraction stimulates both fat synthesis and growth but fractionation experiments indicate that these effects are due to separate fractions.

**Enzymic Oxidation of Cystine to the Corresponding Sulfone.**

BY GRACE MEDES. *From the Lankenau Hospital Research Institute, Philadelphia*

Among the enzymes acting on the sulfur-containing amino acids is one which oxidizes the disulfide group of cystine to the sulfone stage, but does not produce inorganic sulfate. The revised method of preparation is as follows: The liver of the albino rat is perfused with physiological salt solution, ground in sand in acetate buffer at pH 5.2, let stand at 0° for 20 minutes, centrifuged, the supernatant liquid filtered through a sintered glass filter, and the enzyme precipitated with ammonium sulfate between the concentrations of 25 and 40 per cent. The precipitate is taken up in distilled water and dialyzed until sulfate-free.

The rate of oxidation was measured by the rate of disappearance of sulfhydryl plus disulfide. In fifteen experiments with cystine and cysteine as substrates at pH 7.2, sulfhydryl plus disulfide disappeared more rapidly from the solution containing cystine than from that with cysteine, indicating that cystine is the immediate substrate.

The degree of oxidation of the sulfur group, determined by iodometric titration, confirmed the previous finding that oxidation to a sulfone occurs. Amino nitrogen remains unchanged. The carboxyl group, estimated by Mason's manometric method, showed a slight loss, especially in crude extracts. With highly purified preparations, carboxyl is unaltered.

Therefore it is concluded that the enzyme converting cystine to its corresponding sulfone is distinct from the one which decarboxylates it with production of the amine.

**Phosphorylation and Stability of Thiamine in Digestive Secretions.** BY DANIEL MELNICK, WILLIAM D. ROBINSON, AND HENRY FIELD, JR. *From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor*

When thiamine is taken orally by normal subjects, postabsorptive and fasting, the *extra* urinary thiamine is from 10 to 40 per cent of that found when the same test dose is taken with a meal. That this difference in excretion may be due to the instability of thiamine in an alkaline gastrointestinal tract is supported by the exceedingly small urinary thiamine values consistently obtained, despite adequate dietary thiamine intakes, with patients receiving antacid medication and those with achlorhydria. Additional studies have been conducted with thiamine added to aliquots of

human gastric and pancreatic juice and hepatic bile, incubated at varying pH values. The vitamin is stable in gastric juice at a pH from 1 to 7.5. Losses of from 10 to 90 per cent, however, occur when antacids ( $\text{Al}(\text{OH})_3$ ,  $\text{MgCO}_3$ ,  $\text{CaCO}_3$ , or  $\text{Mg}_2\text{Si}_2\text{O}_7$ ) are present due to alkaline destruction and adsorption of the vitamin. In tests with bile and pancreatic juice incubated at their natural pH of 8, only from 20 to 50 per cent of the added thiamine is recovered. More than half of that, which is unaccounted for, is actually destroyed; the remainder is phosphorylated and cannot be determined chemically until after enzymic hydrolysis. Neither preliminary heating at  $100^\circ$  nor the presence of 0.01 M sodium iodoacetate during incubation prevents phosphorylation. After incubation at pH 4.5, however, the added vitamin is recovered quantitatively in the free form. It is concluded that the acidity of the gastric chyme minimizes the destruction of thiamine during absorption. Phosphorylation prior to absorption may occur.

**The Effect of Iodine and Other Mineral Supplements on Cholesterol-Induced Atherosclerosis in Rabbits.** BY HELEN S. MITCHELL, MILDRED F. GOLDFADEN, AND GERTRUDE J. HADRO.  
*From the Nutrition Laboratory, Massachusetts State College, Amherst*

The feeding of cholesterol to rabbits has become an effective means of producing typical atherosclerosis. A prolonged period of hypercholesteremia precedes and is supposed to be partially responsible for the pathologic change. The disagreement in the literature regarding the influence of certain forms of iodine on the level of blood cholesterol and on the severity of the aortic lesions stimulated further investigation.

Rabbits develop marked hypercholesteremia and severe aortic lesions in 6 six weeks with 1 per cent cholesterol in the ration and moderately severe changes with 0.5 per cent cholesterol. Potassium iodide (25 mg. of iodine per 100 gm. of ration) afforded no protection against arterial lesions and tended to increase the hypercholesteremia. Kelp, furnishing a similar amount of iodine, effected a material reduction in both blood cholesterol and arterial damage. A salt mixture containing the excess of basic elements found in kelp plus the same amount of iodine failed to give significant results. Kelp ash which had lost two-thirds of its iodine upon ashing was ineffective. A synthetic salt mixture made to

resemble as closely as possible the inorganic composition of kelp was almost as effective as the original dried kelp, lowering the blood cholesterol and inhibiting the atherosclerotic damage. Thus there would appear to be some inorganic element or elements in addition to iodine essential for the inhibition of injury due to the feeding of cholesterol to rabbits.

**Recovery from Hypervitaminoses D<sub>2</sub> and D<sub>3</sub> As Affected by Dietary Calcium and Phosphorus and Vitamin A Supply.** BY AGNES FAY MORGAN AND NOBUKO SHIMOTORI. *From the Laboratory of Home Economics, University of California, Berkeley*

Irradiated ergosterol and irradiated animal sterol (Delsterol) were fed in doses of 200 U.S.P. units of vitamin D per gm. of body weight per day to young rats on a purified basal diet of normal, high, and low Ca to P ratio and contents. After 28 days some of each group were killed, the vitamin D was removed from the diet of some of each group remaining, and the feeding continued for 28 days longer. All except one group received the same amount of vitamin A from a shark liver oil, 200 units per gm. of body weight per day.

With the basal diet of normal calcium and phosphorus content, by all criteria used the group receiving little vitamin A was more severely damaged by the excess vitamin D than were any of the others. These and other groups given excess vitamin D<sub>3</sub> recovered more rapidly when the overdosage was discontinued than did those receiving excess vitamin D<sub>2</sub> but continued excess vitamin D<sub>3</sub> intake produced more deaths, more soft tissue calcification, poorer growth and bone development than did excess vitamin D<sub>2</sub>. The rats which received the the low calcium diet were favorably affected by excess vitamin D and unfavorably affected by removal of the excesses. Those which received the low phosphorus diet were unfavorably affected by vitamin D excess and recovered promptly on removal of the excess only in the case of vitamin D<sub>3</sub>. Similar effects were noted in young dogs similarly treated. Better absorption and better excretion of excess vitamin D<sub>3</sub> than of excess vitamin D<sub>2</sub> may account for these differences.

**The Effect of Methionine on Normal and Tumor Growth.** BY H. P. MORRIS AND CARL VOEGTLIN. *From the National*



*Cancer Institute, United States Public Health Service, Bethesda, Maryland*

Young normal mice and rats were fed a methionine-deficient diet for 3 and 4 weeks respectively. The animals were then divided into three equal groups; the first group was continued on the basal low methionine diet composed of arachin 15, starch 54.5, salts 4.5, Crisco 21.0, and cod liver oil 5.0 plus growth vitamin concentrates. The second group received a supplement of 450 mg. of *dl*-methionine per 100 gm. of completed diet, while the third group received 362 mg. of *l*-cystine per 100 gm. of diet. Slow growth was obtained on the basal diet with both rats and mice. The cystine-supplemented basal diet permitted slightly better growth than that obtained on the basal diet, while the methionine-supplemented basal diet produced marked stimulation of growth in both mice and rats.

Adult female C<sub>3</sub>H strain mice bearing spontaneous mammary tumors were maintained at constant body weight on this basal diet for 4 weeks. The diet was then supplemented with *dl*-methionine for 3 additional weeks. The average daily growth of the tumors for the methionine-supplemented period was statistically significantly greater than the average daily growth of the tumors during the period on the basal diet.

**Studies on Hematin Metabolism in Dogs.** BY DEMPSIE B. MORRISON, EDWARD F. WILLIAMS, JR., AND W. A. D. ANDERSON. *From the Departments of Chemistry and Pathology, College of Medicine, University of Tennessee, Memphis*

Dogs have been injected intraperitoneally, subcutaneously, or intravenously with solutions of disodium ferrihemate (hematin), of pH 7.6, prepared from recrystallized hemin. Intraperitoneally, the equivalent of 200 mg. of hemin was injected thrice weekly for 7 to 19 weeks; subcutaneously, up to five injections were given on alternate days. Intravenously, ferrihemate was given to produce initial plasma concentrations of 10 to 51 mg. per cent. Absorption from subcutaneous and intraperitoneal routes was very slow. Toxic effects, which may be explained largely by the relative insolubility of ferrihemate at blood pH, varied with concentration, rate of absorption, and method of administration. Ferrihemate injected intravenously persisted in the plasma for several hours to

2 days depending on initial concentration. It was not excreted in the urine and did not form a methemoglobin-like derivative with the plasma proteins. There was an occasional slight increase of bilirubin and porphyrins in urine but no elevation of serum bilirubin. Ferrihemate was carried and deposited in the reticulo-endothelial system. Our results indicate that the dog has a very limited capacity to metabolize unconjugated hematin.

**Morphine Excretion As Determined in Hydrolyzed and Unhydrolyzed Urine from Morphine Addicts.** BY FRED W. OBERST. *From the United States Public Health Service Hospital, Lexington, Kentucky*

The relationship between the amounts of morphine administered and its urinary excretion was investigated in morphine addicts receiving the alkaloidal sulfate subcutaneously in amounts varying from 100 to 4400 mg. per day. Urine saturated with sodium bicarbonate yields morphine base which presumably was combined with acids as salts. Urine acidified with one-fifth of its volume of concentrated hydrochloric acid and hydrolyzed for 3 hours yields from 2 to 21 times more morphine, depending on the amount injected. The additional morphine presumably is conjugated with some unknown substance, since only after long and vigorous acid hydrolysis was the maximal amount of the morphine liberated. The quantitative determination of total morphine in hydrolyzed urine was made by three independent methods. The morphine base was also identified by its isolation from the hydrolyzed urine in pure crystalline form melting at 247-248°.

The amounts of both the free and the combined morphine in the urine were increased when the dosage levels were markedly increased. In 64 samples of urine from eleven patients the average daily per cent of morphine excreted in unhydrolyzed urine was 5.0 (range 0.7 to 12.0), while that in hydrolyzed urine was 30.5 (range 12 to 55). The low percentage excretion values for a given dosage are invariably associated with low urine volumes, and the high values with high urine volumes.

**The Nature of the Blood and Tissue Changes Produced by Feeding Cholesterol to Guinea Pigs.** BY RUTH OKEY AND VERA D. GREAVES. *From the Department of Home Economics and the*

to be extracted from crude concentrates with ether and its stability to alkali.

Fractions containing each factor practically free of the other have been prepared. Neither is active alone but good responses are obtained when the two fractions are combined. However, growth comparable to that obtained with liver extract does not result when more purified fractions of factor W and pantothenic acid are fed, indicating that at least one more growth factor is required by the rat.

**The Effects of Certain Inorganic Substances on the Glucose Tolerance of the Diabetic Rat.** BY JAMES M. ORTEN AND HENRY B. DEVLIN. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

A study of the effects of copper, which has been alleged to exert an insulin-sparing action in the diabetic organism, and of sodium and potassium chlorides on the tolerance to glucose of diabetic rats is being made. Adult intact and partially pancreatectomized rats of the Connecticut Agricultural Experiment Station strain were used as experimental animals. After 16 to 18 hours fasting, 350 mg. of glucose per 100 gm. of body weight were administered intraperitoneally and the sugar content of blood obtained from a tail vein was determined at regular intervals during a 5 hour period.

Approximately 40 per cent of the intact rats were found to have a low tolerance to glucose, the tolerance curves resembling those of the partially pancreatectomized animals. The administration of copper, as copper sulfate, either orally, subcutaneously, or intraperitoneally before or at the time of the injection of the glucose had no appreciable effect on the tolerance to glucose of either the intact diabetic rats or the pancreatectomized animals. However, the administration of sodium chloride, isotonic with the glucose solution injected, resulted in a normal tolerance to glucose in both types of diabetic rats. The results of further studies, now in progress, will be discussed.

**On the Quantitative Determination of Nicotinic Acid and Its Derivatives in Urine.** BY WILLIAM A. PERLZWEIG, EDWARD D. LEVY, AND HERBERT SARETT. *From the Department of*

*Biochemistry, Duke University School of Medicine, Durham, North Carolina*

All of the recent colorimetric methods for the determination of nicotinic acid and its derivatives in biological materials are based either on Vongerichten's reaction with dinitrochlorobenzene\* or Koenig's reaction with CNBr and an aromatic amine.† On application of these methods to urine it was found that human and dog urines contain pyridine derivatives (pyridine, methylated pyridinium bases, nicotine in smokers) which modify the above reactions, interfere with them, and prevent the quantitative recovery of added nicotinic acid. This becomes clearly apparent when the absorption of the color is measured spectrophotometrically throughout the entire visible range. In an attempt to separate the interfering substances from nicotinic acid the following steps were developed: selective adsorption of nicotinic acid and its derivatives upon a fullers' earth and elution; optimal conditions for the complete hydrolysis of the amide and of the glycine conjugate; distillation of the pyridine bases and of alkaloids; removal of the urinary pigments with Pb salts; color development with CNBr and an amine and the extraction of the dye with butyl alcohol.

By combining these steps it has been possible so far to effect only a partial elimination of the interfering factors. The application of rigorous criteria to the solution of this problem will be discussed.

**Biotin Requirements of Clostridia and Assay of Biological Materials for Biotin.** BY W. H. PETERSON, L. E. McDANIEL, AND ELIZABETH MCCOY. *From the Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

Biotin has been reported recently as essential for the growth of certain *Clostridia*. We have confirmed the report of Snell and Williams\* and have tested additional species against either biotin (kindly supplied by Dr. Williams) or a biotin-rich concentrate. On a synthetic medium (glucose, asparagine, and salts) addition of biotin (0.00001 microgram per cc.) produced good growth with

\* *Ber. chem. Ges.*, **32**, 2571 (1899).

† *J. prakt. Chem.*, **69**, 105 (1904).

\* Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, **61**, 3594 (1939).

seven species, including *Clostridium sporogenes*, not previously known to require biotin. These results indicate that at least one constituent of the so called "*sporogenes* vitamin" is biotin.

No response was obtained with twenty other species, of which the following are representative: *Cl. acetobutylicum*, *Cl. botulinum*, *Cl. welchii*, *Cl. histolyticum*. Many of these probably require biotin but failed to grow because of the absence of other essential constituents. Some of the latter are probably unidentified, for in the case of *Cl. acetobutylicum* no combination of known factors induced growth.

Since biotin is the only growth factor required by *Cl. butylicum*, this organism may be used to assay natural materials. The biotin content is ascertained by comparing the growth produced on addition of a natural material to the basal medium with that obtained with known amounts of pure biotin. The richest sources proved to be molasses, Vitab II, alfalfa, and egg yolk. Cereal grains, peptone, and extracts of liver, yeast, beef, and malt were fairly rich. Lean meats, vegetables, fruits, and milk were poor sources. The wide distribution of biotin in plant and animal materials suggests that it may also be required by animals.

### Isolation of a New Nitrogenous Acid from the Adrenal Gland.

By J. J. PFIFFNER AND H. B. NORTH. *From the Research Laboratories of Parke, Davis and Company, Detroit*

While seeking a further supply of the phenolic base, adrenodiamine, we have isolated a new acid from the adrenal glands of cattle. It is very soluble in most organic solvents but crystallizes readily in rosettes of long flat needles from dilute alcohol or from water on acidification of its sodium salt. It melts at 124–125° (uncorrected). The most probable molecular formula is  $C_{12}H_{20}O_3N_2$ . It is optically inactive. Examination of its absorption spectrum indicated a single band at approximately 220 m $\mu$ . With diazomethane it yields a liquid monomethyl ester. A crystalline *p*-phenyl phenacyl ester and *p*-nitrobenzyl ester were prepared. The former crystallizes with 1 molecule of ethyl alcohol and has no sharp melting point. The latter melts at 114–116°. The compound was not acetylated nor did it form a semicarbazone under the usual conditions. It gave a negative test for alkoxyl and was not attacked by warm nitric acid. The substance is seemingly unrelated to the functional activity of the

gland but the elucidation of its structure should prove of interest from a metabolic point of view. We suggest the name "adrenic acid."

**Some New Adrenal Steroid Ketones.** BY J. J. PFIFFNER AND H. B. NORTH. *From the Research Laboratories of Parke, Davis and Company, Detroit*

We wish to add two  $\alpha$ - $\beta$ -unsaturated ketones to the large number of adrenal steroids which have been isolated and described in recent years. One has been shown to be a 17-hydroxyprogesterone. It is practically devoid of progestational or cortical activity but displays androgenic activity comparable to that of adrenosterone in the immature castrated rat. Formation of this compound in the organism may explain the androgenic action of large doses of progesterone. It may also account in part for the androgenic activity of the urine in cases of adrenal tumor. The second ketone (m.p. 261–264° with decomposition, uncorrected;  $[\alpha]_D^{23} = +133^\circ \pm 4^\circ$ ) is of particular interest because results obtained thus far indicate it to be a member of a new series. It analyzes for  $C_{21}H_{28-30}O_4$ . It yields a monosemicarbazone and a monoacetate (m.p. 202–204°). With mild chromic acid oxidation a neutral oxidation product was obtained (m.p. 206–208°) which analyzed as  $C_{21}H_{26-28}O_4$ , and which yielded a monosemicarbazone (m.p. 242–245° with decomposition). These observations indicate that all 4 oxygen atoms are on the nucleus. The carbonyl group is presumably in the 3 position. The reactive hydroxyl group may be located at C 11 with a steric configuration opposite to that in corticosterone. This would account for the formation of a neutral oxidation product having only one reactive carbonyl group. The compound has no cortical, androgenic, or progestational activity.

**The Excretion of Sulfate by Normal Men.** BY MARSCHELLE H. POWER, ARNOLDUS GOUDSMIT, JR., AND NORMAN M. KEITH. *From the Division of Biochemistry and the Division of Medicine, The Mayo Foundation, Rochester, Minnesota*

We have reported previously\* that when solutions containing both sodium sulfate and sucrose are injected intravenously the

\* Power, M. H., Goudsmit, A., Jr., and Keith, N. M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. lxxix (1939).

sulfate clearances, which at endogenous levels of the serum sulfate are about 25 to 40 per cent of sucrose clearances, increase to values nearly equal to or sometimes slightly greater than the sucrose clearances. The sulfate to sucrose clearance ratios in a given individual were found to be constant as the serum sulfate decreased from values 15 to 30 times normal down to values 7 to 10 times normal. Subsequent data indicate that within the range of serum sulfate from normal to about 5 times normal the sulfate clearances will assume values intermediate between the endogenous and the maximal clearances, depending on the level of the serum sulfate.

Our results are in harmony with those published recently by Bjering and Øllgaard.† These authors accounted for the relatively low endogenous sulfate clearances in man by assuming that most of the inorganic sulfate is present in plasma in a non-filtrable form, and conversely, for the relatively high clearances observed after administration of sulfate by assuming that exogenous sulfate is freely eliminated by glomerular filtration without appreciable tubular reabsorption. We consider that the view of non-filtrability of endogenous plasma sulfate is less well supported by experimental evidence than that of practically complete filtrability; consequently we retain the opinion that endogenous and exogenous sulfates are both probably excreted by glomerular filtration with subsequent reabsorption of more or less constant amounts of the filtered sulfate.

**Assay Method for Vitamin K.** BY ARMAND J. QUICK. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

Since vitamin K is essential for the synthesis of prothrombin, the most logical method for assaying this agent is to find the minimum amount required to restore the prothrombin to the normal level in chicks suffering from a deficiency of vitamin K. The following method was developed. Chicks 1 week old are put on the Almquist diet. When the prothrombin is decreased to 10 per cent of normal, the material to be tested is fed. After 24 hours, the prothrombin of the blood is determined. The smallest quantity necessary to restore the prothrombin to the normal level

† Bjering, T., and Øllgaard, E., *Acta med. Scand.*, 102, 55-78 (1939).

can readily be determined. For the estimation of prothrombin, 0.45 cc. of blood is taken from the wing vein and mixed with 0.05 cc. of 0.1 M sodium oxalate. The thromboplastin is prepared from chicken brain by the author's acetone method. The same technique is used for determining prothrombin in chicken as in human blood. By means of this method the maintenance dose of vitamin K can be determined as well as the daily loss of prothrombin. It is further useful for demonstrating the greater efficacy of vitamin K over transfusion for restoring prothrombin. Feeding excessive amounts of vitamin K will not cause the prothrombin to rise above a fixed level.

**Further Studies on the Determination of Inorganic Sulfate in Serum.** BY JOHN G. REINHOLD AND T. V. LETONOFF. *From the Division of Biochemistry, Philadelphia General Hospital, and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

When sulfate is added to serum preceding deproteinization by uranium acetate as described in the method of the writers\* or by the ferric chloride-ammonium hydroxide-ammonium acetate reagent of Hoffman and Cardon,† recovery is incomplete unless protein is removed immediately. About 40 per cent of the sulfate added is not recovered, variations in the quantities added having little effect over wide limits. However, sulfate added to citrated plasma can be recovered quantitatively for an indefinite period. Binding of the added sulfate by serum therefore is associated with the clotting mechanism and the presence of only a small clot prevents quantitative recovery of added sulfate. The possible relationship to heparin or other sulfur-containing anticoagulant is to be investigated.

Despite the reaction of sulfate with serum, serum and citrated plasma derived from the same specimen of blood give identical values for inorganic sulfate. That portion of the sulfate added to serum not recovered as inorganic sulfate cannot be recovered in the total non-protein sulfur of filtrates of the type mentioned above. It is liberated when serum is deproteinized by trichloroacetic acid. However, trichloroacetic acid, especially in higher

\* Letonoff, T. V., and Reinhold, J. G., *J. Biol. Chem.*, **114**, 147 (1936).

† Hoffman, W. S., and Cardon, R., *J. Biol. Chem.*, **109**, 717 (1935).



concentrations (1 cc. of 20 per cent solution per cc. of serum), attacks also ethereal sulfate and liberates significant amounts of sulfate; therefore its suitability when used in such concentrations for the determination of inorganic sulfate of serum is questionable.

**The Relationship of Phosphorus to the Metabolism of Fat and Glucose.** BY RAYMOND REISER. *From the Animal Husbandry Research Laboratory, State College of Agriculture, University of North Carolina, Raleigh*

The intestinal mucosa, liver, and kidney of fasting swine were analyzed 1 hour after the ingestion of glucose and 2 hours after the ingestion of cottonseed oil. It was found that after the ingestion of glucose the acid-soluble ester phosphorus of the mucosa and kidney increased, the increase in the mucosa being partially at the expense of the inorganic fraction. Both the inorganic and ester fractions of the acid-soluble phosphorus of the liver decreased. The inorganic fraction of the kidney did not change significantly.

After the ingestion of oil there was a marked increase in the acid-soluble ester phosphorus of all three organs and a smaller though significant increase in the inorganic phosphorus of the mucosa and liver. There was, also, an increase in the total reducing substance in the trichloroacetic acid filtrate of all three organs after oil ingestion, the increase in the liver being as great as after glucose.

The data have been evaluated statistically.

**Liquid Ammonia As a Reagent in the Study of Biochemical Compounds.** BY RICHARD G. ROBERTS. *From the Department of Physiological Chemistry, Chicago Medical School, Chicago*

Liquid ammonia is the most general solvent known, second only to water.\* However, most of the work on this nitrogenous solvent has been done with salts and simpler organic compounds. But liquid ammonia is also a good solvent for many biological compounds of complex structure. Here, however, with compounds of definite physiological activity, such as the hormones, two other factors became of great importance, inactivation and prolonged or intensified activity.

\* Franklin, E. C., Nitrogen system of compounds, New York (1935).

Most of the amino acids and some simple peptides are soluble in liquid ammonia, while most proteins are insoluble, crystalline insulin being a notable exception. Adrenalin forms a suspension, but passes through a sintered glass filter of 20  $\mu$  porosity, forming a clear filtrate which reverts to a suspension on standing. It seems to exhibit properties of thixotropy, and precipitates from liquid ammonia in the form of circular platelets. It forms a precipitate with glycine, which has a more prolonged pressor action than adrenalin. Crystalline insulin forms a precipitate with glycine also, but the product, if anything, is less active. Hematin is soluble and forms a conjugate with glycine, this conjugate being soluble in water at pH 7.4. Parathyroid hormone is insoluble and slightly inactivated. Beef and hog anterior pituitary powders yield soluble fractions, all being inactive; gonadogen (Upjohn) does likewise. Powdered digitalis yields a soluble fraction, both fractions being inactive. Powdered senna also yields a soluble fraction, but the activity remains in the insoluble fraction. (With the exception of adrenalin, the bioassay determinations were made by Dr. W. R. Tweedy, Dr. E. Geiger, and Dr. C. P. Kraatz.)

## The Biuret Reaction in the Determination of Serum Proteins.

**I. A Study of the Conditions Necessary for the Production of a Stable Color Which Bears a Quantitative Relationship to the Protein Concentration.** BY HOWARD W. ROBINSON AND CORINNE G. HOGDEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

Although the biuret reaction has been successfully employed for the determination of serum proteins, many investigators have avoided its use because of unsatisfactory standards, instability of color, and presence of other color-producing substances.

Absorption curves of the color produced with varying concentrations of NaOH and CuSO<sub>4</sub> have been made and the density values at wave-lengths 560 and 700 m $\mu$  compared with the nitrogen content of the solution. A color which remained constant for at least 48 hours was obtained in this manner: Serum is precipitated with CCl<sub>3</sub>COOH, centrifuged, the precipitate dissolved in 3 per cent NaOH, and color developed with CuSO<sub>4</sub>. A wide

variation in the concentration of Cu had no influence on color. Rabbit serum diluted with saline was more satisfactory as a standard than dog or human serum, for, when kept in a refrigerator for 6 months, it gave the original protein value. In 3 per cent NaOH the density values were entirely proportional to the quantity of protein present and the amount due to Cu uncombined with protein was almost negligible, whereas in higher concentrations of alkali this increment became a much greater proportion of the total density.

Filtration of the color solution through No. 00 Munktell paper lowered the density value with no change in the nitrogen content. Reproducibility of this clear color solution in relation to N content has led us to believe that accurate determinations can be made without a standard in any photoelectric colorimeter with suitable filters.

**The Relation of Nutrition to Gastric Function. II. The Effect of a Dietary Deficiency of Vitamin C.** BY JOSEPH H. ROE, JAMES M. HALL, AND HELEN M. DYER. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*

Guinea pigs were placed on a scurvy-producing diet. In determination of the gastric function histamine was injected into a fasted animal every 15 minutes and the stomach contents were removed every half hour for 2 hours. The volume of gastric juice, the total acidity, and the free HCl were determined. In acute scurvy there is a marked reduction in the volume of gastric juice secreted and a decrease in the acidity. Up to the time of onset of severe scurvy symptoms there appears to be little or no diminution in secretory function.

**Chemical Studies on Powdered Wool.** BY JOSEPH I. ROUTH. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Several batches of powdered wool were prepared by being ground in a ball mill for variable periods of time. Before it was ground, the wool was either cut into short fibers with scissors or into very fine pieces with a Wiley mill. The powdered material was separated into fractions of different particle size by a series of screens (100 to 325 mesh).

By extraction with water each of these fractions was separated into soluble and insoluble portions. Nitrogen, sulfur, and cystine determinations were made on the original powdered wool, on the water-soluble portion, and on the residual insoluble material.

The nitrogen and sulfur content of the wool was not appreciably altered by the grinding process; however, a progressive decrease in cystine was observed as the time of grinding increased. The soluble portion contained nitrogen and sulfur, which increased with the time of grinding. It appears that the duration of grinding has more influence on the increase in soluble nitrogen and sulfur of the powdered wool and on the decrease in its cystine content than the variation in particle size.

**The Supplementary Relationship of Vitamin B<sub>6</sub> and Unsaturated Fatty Acids.** BY W. D. SALMON. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

On a fat-free diet supplemented with carotene, calciferol, thiamine, riboflavin, and factor 2, rats made very little growth and consistently developed erythema on the nose and feet, a generalized scaliness and fissuring of the skin, and a severe hematuria. The addition of synthetic vitamin B<sub>6</sub> (Merck) produced a marked stimulation of growth, prevented the erythema, and reduced the severity of the scaly condition of the skin. The hematuria was not prevented, however, and the rats eventually succumbed.

The addition of methyl linolate or corn oil (without vitamin B<sub>6</sub>) prevented the hematuria as well as the erythema and scaliness of the skin but produced relatively little growth. The further addition of vitamin B<sub>6</sub> resulted in a marked increase in the rate of growth.

When the carbohydrate of the vitamin B<sub>6</sub>-free diet was entirely replaced with fat, a slow but continued rate of growth resulted. The addition of vitamin B<sub>6</sub> to the high fat diet markedly increased the rate of growth.

Although the vitamin B<sub>6</sub> and the essential fatty acids can to a certain extent mutually alleviate the deficiency of each other, the presence of both in the diet is necessary for normal nutrition of the rat.

**Studies on Invertebrate Hemoglobins.** BY KURT SALOMON. *From the Laboratories of Physiological Chemistry and of Pharma-*

*cology and Toxicology, Yale University School of Medicine, New Haven*

Two hemoglobins occurring in worms (erythrocruorins in Svedberg's terminology) have been studied from a chemical and physicochemical point of view in order to enable a comparison of their properties with those of vertebrate hemoglobin. These properties include the absorption spectrum of the pigments and of some of their derivatives, their stability towards denaturation, the ease of their oxidation to the ferri compounds, their behavior in the ultracentrifuge and in electrical fields. A successful attempt has been made to utilize the air-driven ultracentrifuge for the purification of such pigments. Particular attention was paid to the question as to the number of heme groups per erythrocruorin molecule. Two very different types of erythrocruorin were studied in this work, *viz.* the macromolecular respiratory pigment of the common earthworm (*Lumbricus terrestris*) and the low molecular respiratory protein of the so called blood-worm (*Glycera dibranchiata*, Ehlers). In accordance with the general rule of Svedberg the former is freely dissolved in the plasma, whereas the latter is locked up in corpuscles which are found in the body fluid.

The relatively large amount of pigment present in the blood-worm has made it possible to isolate sufficient quantities of pure crystalline hemin to permit a determination of the configuration of the porphyrin side chains in order to decide whether the heme grouping in invertebrates is identical with that of the vertebrates as is generally assumed without a sufficient experimental basis.

**Direct Action of Nicotinamide As a Respiratory Stimulator.** By FELIX SAUNDERS, ALBERT DORFMAN, AND S. A. KOSER. *From the Departments of Biochemistry and of Bacteriology and Parasitology of the University of Chicago, Chicago*

The respiration of dysentery bacilli grown on suboptimum amounts of nicotinamide with glucose as substrate is stimulated by nicotinic acid, nicotinamide, or coenzyme I. This stimulation might be thought of as being due to synthesis of coenzyme I or II, or a closely related coenzyme. The purpose of this report is to show that nicotinamide functions directly as a respiratory stimulator without being synthesized to a known coenzyme. This

statement is supported by the following experimental results. (1) Coenzymes I and II are less effective as growth factors for dysentery bacilli than an equivalent amount of nicotinamide. If coenzymes I and II are autoclaved their growth-promoting activity is increased. (2) The ratio, time of reduction of methylene blue by cells to reduction by cells plus nicotinamide, is not affected by time of incubation. (3) Coenzymes I and II are less active than nicotinamide as respiratory stimulators. When coenzymes I and II are autoclaved, their activity is increased. The more complete the hydrolysis, the greater the increase in activity. (4) When *Proteus vulgaris* cells grown on a medium deficient in nicotinamide are used, nicotinamide stimulates the respiration with glucose as substrate, but with lactate as substrate nicotinamide is inactive, while cozymase stimulates respiration. Other systems which are known to be stimulated by coenzyme I are not stimulated by nicotinamide. On the basis of these facts we postulate that metabolism of glucose by dysentery bacilli involves a type of respiration not yet described and one in which nicotinamide can act directly without previous synthesis to a known coenzyme.

**Development of "Sulfapyridine Fastness" in Vivo.** BY L. H.

SCHMIDT AND HERMAN DETTWILER. *From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati*

A group of mice was infected with a strain of Type III pneumococcus and was treated with sulfapyridine for 6 days. All of these mice survived the treatment period, but died within 3 days after therapy was stopped. A second group was infected similarly with pneumococci isolated at the time of death from mice belonging to the first group. These mice were treated with sulfapyridine as were those of the original group, but some died during the treatment period and all were dead within 36 hours after therapy was discontinued. When this infection procedure was repeated in a third, fourth, and fifth group of mice, deaths occurred earlier in each succeeding group. In the fifth group some of the animals died as early as the 2nd day of treatment, the majority died on the 3rd day, and all were dead by the 4th day. Since there were no differences between the virulence and invasiveness of the parent organisms and those isolated from mice of the fifth treated group,

the pneumococci must have become insensitive or "fast" to the drug during the treatment periods.

The results of experiments *in vitro* support this conclusion. Sulfapyridine added to infusion broth, or infusion broth plus glucose, or blood broth, inhibited the growth of the parent organisms throughout a 48 hour period. Under similar conditions the growth of the "fast" strain was inhibited for 12 hours only, after which this strain grew as well in sulfapyridine-containing media as in media without sulfapyridine.

**Evidence of the Complex Nature of the Alcoholic Precipitate Factor Required by the Chick.** BY A. E. SCHUMACHER, G. F. HEUSER, AND L. C. NORRIS. *From the Department of Poultry Husbandry, Cornell University, Ithaca*

A report was made recently of a factor, precipitated by alcohol, which is required for growth and reproduction in the domestic fowl. In order to study the alcohol precipitate factor more effectively a special diet,\* more deficient in the factor than the ones previously used, was developed. This diet, it is now evident, is deficient in at least two factors required for chick growth rather than one.

The factors are extracted from yeast with 0.24 N HCl. Upon concentration and addition of 10 parts of ethyl alcohol by volume, factor 2 is precipitated, while factor 1 remains in the filtrate. Factor 1 is then precipitated by neutralizing the alcohol filtrate.

The addition of factor 2 equivalent to 5, 10, and 15 per cent yeast to the basal diet promoted growth which was significantly better than that obtained with the unsupplemented diet. No differences in growth were obtained on the various levels of factor 2. The addition of factor 1 equivalent to 5 per cent yeast to factor 2 promoted growth which was significantly better than that obtained on factor 2 alone. This growth was equal to that obtained with the untreated extract equivalent to 5 per cent yeast.

By precipitation with alcohol both factors are separated from nicotinic acid, pantothenic acid, riboflavin, thiamine, and vitamin B<sub>6</sub>. Differences in solubility favor the non-identity of these factors with factor W. Their presence in yeast makes it improbable that either one is identical with the grass juice factor.

\* Schumacher, A. E., and Heuser, G. F., *Poultry Sc.*, 18, 411 (1939).

**Ascorbic Acid and the Metabolism of the Aromatic Amino Acids, Phenylalanine and Tyrosine.** BY ROBERT RIDGELY SEALOCK, JESSE D. PERKINSON, AND HANNAH E. SILBERSTEIN. *From the Department of Vital Economics, The University of Rochester, Rochester, New York*

In the recent demonstration that vitamin C prevents the excretion of homogentisic acid resulting from the feeding of tyrosine to guinea pigs, qualitative tests indicated that other metabolites were also excreted on the ascorbic acid-deficient diet. With daily supplements of 0.5 to 1.0 gm. of *L*-tyrosine approximately 30 per cent is present in the urine as homogentisic acid and 30 per cent as the *p*-hydroxyphenylpyruvic acid. With similar amounts of *L*-phenylalanine comparable levels of the corresponding metabolites are present. In addition, traces of the lactic acid derivatives and the unchanged amino acids are also excreted. Administration of 5 to 10 mg. of ascorbic acid daily inhibits within 24 to 72 hours the excretion of the catabolites and subsequent removal of the vitamin causes their prompt reappearance in the urine. That the state of vitamin saturation of the animal body is an important factor is indicated by the fact that animals exhibiting scorbutic symptoms require larger doses of vitamin than those with no symptoms. These findings with experimental animals furnish evidence that ascorbic acid is essential to the normal oxidative breakdown of these aromatic amino acids and are in agreement with the recent experiments of Levine, Marples, and Gordon,\* who find that vitamin C will prevent the excretion of the same metabolic substances by premature infants.

In addition to the above results, similar feeding experiments with the unnatural isomers of phenylalanine and tyrosine and the corresponding keto acids will be reported.

**Studies on the Chemical Treatment of Tumors. V. Separation of the Hemorrhage-Producing Fraction from *Bacillus prodigiosus* Filtrates.** BY M. J. SHEAR AND FLOYD C. TURNER. *From the National Cancer Institute, United States Public Health Service, Bethesda, Maryland*

The method used\* for separating, from broth cultures of *Bacillus*

\* Levine, S. Z., Marples, E., and Gordon, A. H., *Science*, **90**, 620 (1939).

\* Shear, M. J., and Andervont, H. B., *Proc. Soc. Exp. Biol. and Med.*, **34**, 323 (1936).



*coli*, the fraction which produces hemorrhage in mouse tumors was applied to similar cultures of *Bacillus prodigiosus*, an organism employed in the preparation of Coley's "toxins."

Untreated broth culture filtrates contained 330 mouse tumor units per cc. as contrasted with 100 units per cc. for a commercial preparation of "Coley's mixed toxins." (A mouse tumor unit is defined as the amount required for production of hemorrhage in a mouse sarcoma.) Fractionation gave a concentrate containing 20,000 mouse tumor units per cc.

Since the chief impurities were the constituents of the original broth culture medium, simple synthetic media were tried. One consisting of  $\text{NH}_4\text{Cl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{NaOH}$ , and glucose was found satisfactory.

The filtrates, which had the same potency as those from broth cultures, were treated with  $\text{CHCl}_3$ ; the resulting emulsions contained practically all of the active material. After removal of the chloroform from the emulsions, addition of  $\text{C}_2\text{H}_5\text{OH}$  gave a precipitate which was water-soluble. A solution, with a potency of 133,000 mouse tumor units per cc., contained 26.4 mg. of total solids per cc.; i.e., 0.2  $\gamma$  per mouse tumor unit as compared with 0.4  $\gamma$  previously obtained with the *Bacillus coli* concentrate.

The lethal dose of the various concentrates ranged from 100 to 1000 times the minimum effective dose.

Between pH 1 and 10, none of the active agent dialyzed through cellophane; none was destroyed except at high acid concentration.

Techniques for large scale preparation of highly potent concentrates have been worked out.

**pH Relations in Salt-Catalyzed Carbohydrate Oxidation.** By  
FAY SHEPPARD, I. S. DANIELSON, AND MARK R. EVERETT.  
*From the Department of Biochemistry, University of Oklahoma  
Medical School, Oklahoma City*

The theory that carbohydrates function as anions and are activated by hydroxyl ions during direct oxidation is not consonant with the anionic nature of common laboratory oxidants nor with the rapid salt-catalyzed oxidations by bromine and hydrogen peroxide in neutral or slightly acid solutions at room temperatures. Using a MacInnes glass electrode, the authors have made pH measurements in 1 per cent *d*-gluconolactone solutions during salt-

catalyzed oxidation by 2 equivalents of hydrogen peroxide. Only small changes of pH occur during the reaction which is rapid at pH 4 with iron salts. With potassium bicarbonate, oxidation is rapid at pH 6.6, optimal at 6.9 to 7.15, slow at 8.5, and inhibited at slightly greater alkalinity. Oxidative influences of hydroxyl ions are apparently exerted upon the oxidants and salts.

Potassium bicarbonate exhibits stoichiometric relations to gluconolactone; increments in reducing products of the oxidation are 2, 17, 1, and 15 (as per cent glucose) for the first 4 equivalents of bicarbonate respectively, and 1 per cent each for the next 4 equivalents. This suggests that two pairs of bicarbonate molecules combine with gluconolactone to effect catalysis. Phosphates are inactive *per se* but 2 equivalents of dipotassium phosphate act synergistically with 4 of potassium bicarbonate to give 22 per cent additional reducing material at pH 7.15. Copper, iron, nickel, and tungstate ions also catalyze hydrogen peroxide oxidation of carbohydrates. Dipotassium phosphate is inhibitory to iron salt catalysis. The relative inactivities of alkaline earths, borates, phosphates, etc., are not due to pH variations.

**Effect of Certain Fats upon the Utilization of Carotene.** By W. C.

SHERMAN. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

Young rats receiving a low fat, vitamin A-free diet until ophthalmia and cessation of growth occurred showed a better growth response to low levels of  $\beta$ -carotene when fed with certain purified oils than when fed alone. Oils high in unsaturated fatty acids gave the best growth. Fats which were too low in unsaturated fatty acids to protect against scaliness in the animals had no effect upon the growth.

When methyl linolate was fed with low levels of carotene, the scaliness was alleviated, but the vitamin A deficiency was intensified. After temporary improvement, the ophthalmia recurred and was accompanied by a loss of weight. This interference of methyl linolate with carotene utilization was prevented by the addition of soy bean oil or by feeding the two supplements several hours apart, in which case the methyl linolate improved the growth. Growth was also increased by methyl linolate when it was fed with larger amounts of carotene.

Methyl linolate did not cause direct destruction of the carotene prior to its ingestion by the rats and did not decrease the efficiency of absorption of carotene. It appears, therefore, that linoleic acid interferes with the metabolism of carotene or vitamin A and that certain oils contain factors which protect against this interference.

**The Photoelectric Measurement of Cell Volume.** BY ALFRED T.

SHOHL. *From the Department of Pediatrics, Harvard Medical School, Boston*

A method is presented in which the transmission of light of 660  $\mu$  through a suspension of blood is correlated with values for cell volume obtained by the hematocrit method. For normal blood the correlation lies within 2 per cent. Fluctuations in hemoglobin content are without effect, but pathological variations in the cell size and shape give divergences which are a measure of this variation.

**The Influence of Some Amino Acids on the Production of Fatty**

**Livers in Mice by Diet.** BY SAM A. SINGAL. *From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

The effects of the betaine of cystine, cystine disulfoxide, pentocystine, and hexocystine on the liver fat of mice when added to a basal diet high in fat and low in lipotropic factors (5 per cent arachin and 40 per cent lard) have been studied. The betaine of cystine, like methionine but unlike cystine, has been found to exert a lipotropic action in that it prevented the accumulation of fat in the liver. On the other hand the addition of cystine disulfoxide resulted in the deposition of fat in the liver in amounts over and above those observed on the basal diet, resembling cystine in this respect. It had been shown previously that homocystine could produce an effect similar to that of cystine, but the next higher homologues, pentocystine and hexocystine, were entirely without influence on the production of fatty livers.

**Further Evidence of the Mode of Action of Vitamin D.** BY

MARGARET CAMMACK SMITH AND HARRY SPECTOR. *From the Agricultural Experiment Station, the University of Arizona, Tucson*

That vitamin D functions at least in part by increasing the

permeability of the intestinal canal to the absorption of calcium and phosphorus is indicated by the finding that mineral oil ingestion, which experimental work in this laboratory has shown to interfere with the healing of rachitic lesions in rats by cod liver oil, and to prevent normal retention of calcium and phosphorus by puppies given an adequate ration and supposedly protective doses of vitamin D as cod liver oil by mouth, also interferes with the action of vitamin D which is formed by external irradiation of the animal body.

Rats made rachitic on Steenbock's Diet 2965 were divided into two groups matched as to litter, sex, weight, and handling, except that one group received the basal ration plus 10 per cent mineral oil. Rats from each group were exposed to the ultraviolet lamp at the same time and for the same length of time during a 10 day test period. Line test findings on the bones showed, without exception, that healing was absent or less advanced in the group fed mineral oil. As mineral oil is probably not absorbed and plays its rôle only in the intestinal canal, evidence is given concerning one probable action of vitamin D as an antirachitic agent.

**A Study of Ascorbic Acid Synthesis by Rat Tissue.** By C. V. SMYTHE AND C. G. KING. *From the Department of Chemistry, University of Pittsburgh, Pittsburgh*

Recent papers from our laboratory have shown that the synthesis and excretion of ascorbic acid by albino rats can be greatly stimulated by the administration of any one of a series of organic compounds. The site of formation of the vitamin and the mechanism by which it is formed are entirely unknown. In the present work experiments were carried out with the Warburg technique, on tissue slices and homogenized tissue from normal rats and from rats that were excreting large amounts of ascorbic acid, owing to treatment with the compounds mentioned above. As a general result of treatment with such compounds, there was a decreased rate of glycolysis and of oxygen consumption. The various tissues were analyzed for ascorbic acid at the beginning and at the end of the manometric experiments in the presence of various substrates. Practically all tissues showed an increase in reducing material when held anaerobically, but in some cases it was possible to show by the rate of reduction of dichlorobenzene indophenol that there was practically no increase in ascorbic acid.

In other cases a reducing material was formed that so far cannot be distinguished from ascorbic acid.

**Pantothenic Acid Content of Tissues of Chicks on Diets Deficient in This Substance.** BY E. E. SNELL, DERROL PENNINGTON, AND ROGER J. WILLIAMS. *From the Department of Chemistry, University of Texas, Austin*

Day-old chicks were fed a starting mash for 4 days. One group was then fed Jukes' heated ration.\* A control group was fed this ration supplemented with 0.03 per cent of a pantothenic acid concentrate potency 600.† Jukes found this level sufficient for maximal growth on the above diet.\* Differences began to appear within a week; after 23 days chicks of the deficient group averaged 99 gm. and showed moderate to severe symptoms of chick dermatitis. Normal chicks of the control group averaged 215 gm.

Chicks from each group were killed, and the desired tissues removed, ground, and autolyzed at 37°. Water extracts of the autolyzed tissues were assayed for pantothenic acid by the yeast growth method of Williams *et al.*† and a modification of the bacterial test of Snell *et al.*‡ Results of both tests were in essential agreement. In every case both the amounts and the percentages of pantothenic acid in tissues from the deficient group were decidedly lower than in those from the sufficient group. With spinal cord, brain, leg muscle, and blood the differences exceeded 50 per cent; with liver and kidney deficient tissues contained about 65 per cent as much pantothenic acid as the control tissues.

**Relation between Serum Magnesium Partition and Thyroid Gland.** BY LOUIS J. SOFFER, EDWARD GROSSMAN, AND HARRY SOBOTKA. *From the Laboratories of the Mount Sinai Hospital, New York*

The total magnesium in human blood serum shows but slight and insignificant variations in a variety of diseases; however, the amount of magnesium in an ultrafiltrate from serum varies con-

\* Jukes, T. H., *J. Biol. Chem.*, **129**, 225 (1939).

† Williams, R. J., *et al.*, *J. Am. Chem. Soc.*, **60**, 2719 (1938); *Biochem. J.*, **28**, 1887 (1934).

‡ Snell, E. E., *et al.*, *J. Bact.*, **38**, 293 (1939); *Biochem. J.*, **31**, 1789 (1937).

siderably. Whereas normally 80 to 95 per cent of the total magnesium is not bound by the colloidal constituents of the serum, this figure regularly drops below 80 to values as low as 40 per cent in untreated cases of Graves' disease; on the other hand in myxedema the magnesium is completely filtrable. Administration of iodine as well as thyroidectomy alters the level of ultrafiltrable magnesium.

The values of total and filtrable magnesium in a number of warm- and cold-blooded species have been established as a basis for the investigation of the effect of thyroid and pituitary preparations on the magnesium partition.

**Ketosis Caused by Overdoses of Insulin.** BY MICHAEL SOMOGYI.

*From the Laboratory of the Jewish Hospital of St. Louis, St. Louis*

Insulin can exert two opposite effects upon ketosis. After injection it first lowers the ketonemic level; if, however, it suffices to cause protracted hypoglycemia, ketonemia eventually rises appreciably above the pre-insulin levels. The phenomenon occurs in healthy as well as in diabetic individuals; in the latter, on a greatly magnified scale.

With Hubbard's method, the only reliable micromethod available, we found in healthy men 30 to 50 per cent increases of ketonemia several hours after injecting 12 units of insulin. A 20 to 30 per cent drop preceded the rise.

Similar observations were made on non-diabetic patients who were treated for dementia precox with massive doses of insulin. Increases in ketonemia up to 200 per cent above the pre-insulin levels were found.

The phenomenon was first observed in unstable diabetics; subsequently it was experimentally produced in well regulated patients, who in the past had been quite unstable. Their diets before and during the experiments contained over 300 gm. of available carbohydrate, of which 0 to 15 gm. was lost in the urine in 24 hours. Their insulin dosage was about 20 units per day. After a period of preliminary observation the insulin dose before the evening meal was increased by 6 units; this caused hypoglycemia between 10 p.m. and 3 a.m., invariably entailing substantial degrees of hyperketonemia and ketonuria during the following morning.

**Lipid Metabolism in Brain and Other Tissues of the Adult Rat.**

By WARREN M. SPERRY AND HEINRICH WAELSCH. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Departments of Biochemistry and Neurology, College of Physicians and Surgeons, Columbia University, New York*

The concentration of deuterium was determined in the unsaponifiable lipids and fatty acids of the brain, liver, intestine, and remaining carcass of adult male rats at varying intervals (a) after administration of a single, small dose of deuterium-labeled fat or (b) after enrichment of the body water with deuterium. With procedure (a) traces at the most of labeled fatty acids were found in brain. The highest concentrations were in liver and intestine. A large but variable proportion of the total amount given was in the depot fat.

With procedure (b) the concentration of deuterium in brain unsaponifiable material was small, while that of all other tissues was large. In brain fatty acids on the other hand the concentration was comparable with that found in the depots, and indicated that as much as one-fifth may be replaced in a week. From the large concentrations of deuterium found in the liver fatty acids it was estimated that they may all be replaced within 4 days (the shortest period studied). The concentration in the liver unsaponifiable fraction was on the average less than that of the carcass and no greater than that of the intestine. Thus the liver does not appear to have the same dominant rôle in the metabolism of unsaponifiable substances as it does in fatty acid metabolism. Large concentrations of deuterium in both the unsaponifiable and fatty acid fractions of the intestine direct attention to this organ as an active participant in endogenous lipid metabolism.

**x-Ray Analysis of Protein Denaturation.\*** By MONA SPIEGEL-ADOLF AND GEORGE C. HENNY. *From the Department of Colloid Chemistry, D. J. McCarthy Foundation, and the Department of Physics, Temple University School of Medicine, Philadelphia*

Significant physicochemical differences in serum albumin de-

\* Aided by a grant from the Committee on Radiation of the National Research Council to Mona Spiegel-Adolf.

natured by heat and short wave light have been established before (Spiegel-Adolf). x-Ray diffraction analysis seems particularly useful for the further elucidation of these problems as an interference through changes in solubility and dispersion is excluded.

Electrolyte-free samples of 1 per cent serum albumin solutions were submitted either to a 15 minute boiling or to 4 to 6 hours irradiation with the arc of a mercury quartz lamp, while the temperature did not rise above 29°. The precipitates were washed and dried in a vacuum over  $P_2O_5$ . No appreciable changes in the N content of either the heat-denatured or the light-denatured protein were observed.

The x-ray diffraction patterns of these preparations were studied by the help of the photoelectric microdensitometer of Spiegel-Adolf and Peckham. The relative light transmission graphs indicate that for normal as well as for the denatured proteins the main spacings are identical. The corresponding graph of the light-denatured protein is uniformly lower than the one of the normal product. The graph of the heat-denatured protein comes between these two. But the heat-denatured protein shows three distinct additional rings at distances corresponding to planar spacings of 4.02, 3.73, and 3.49 Å. similar to the one additional ring described by Astbury and Lomax. Treatment of the heat-denatured protein with NaOH restores the x-ray diffraction pattern of the latter to the original shape. Nevertheless the increased light transmission of the diffraction film of the NaOH-treated heat-denatured serum albumin seems to preclude a case of complete reversal of heat denaturation.

**x-Ray Analysis of Tuberculin Proteins.\*** BY MONA SPIEGEL-ADOLF, FLORENCE B. SEIBERT, AND GEORGE C. HENNY. *From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University School of Medicine, the Henry Phipps Institute, University of Pennsylvania, and the Department of Physics, Temple University School of Medicine, Philadelphia*

The importance of x-ray analysis for the elucidation of protein structure seems to warrant the use of this method in biologically active material. The products used and their chemical and physicochemical properties have been described by Seibert. The x-ray diffraction apparatus and technique used have been reported



by Spiegel-Adolf and Henny. The x-ray diffraction patterns of twelve different tuberculin proteins can be divided into three groups.

The presence of phosphate salts with the protein is not evident in the characteristic diffraction lines when the salt concentration is 5 per cent of the total mixture, but becomes evident when it reaches 14 per cent.

Nucleic acid contamination of the tuberculin proteins has been described by Spiegel-Adolf and Seibert. In cases in which the nucleic acid content reaches 17 per cent the diffraction pattern is markedly different from the pure samples and the nucleic acid pattern becomes evident. After removal of the nucleic acid from the tuberculin proteins the diffraction pattern becomes very similar to the ones of the pure products.

The tuberculin proteins give x-ray diffraction patterns very much like the ones of the serum proteins. Differences seem to exist in the diameters of the largest diffraction ring of the various tuberculin preparations. In addition there are differences in the width and the sharpness of the diffraction rings which seem to indicate variations in denaturation and polymerization.

**The Fatty Acid Metabolism of the Liver of the Diabetic Cat.** BY  
WILLIAM C. STADIE, JOHN A. ZAPP, JR., AND FRANCIS D. W.  
LUKENS. *From the Department of Research Medicine and the  
George S. Cox Research Institute, University of Pennsylvania,  
Philadelphia*

According to the Knoop hypothesis,  $\beta$ -oxidation of fatty acid chains in the liver of the diabetic stops at the 4-carbon stage with the resultant formation of large amounts of ketone bodies and acetic acid. We have previously given evidence\* to show that fatty acids are oxidized by the liver of the diabetic cat by multiple alternate oxidation; accordingly, no acetic acid is formed. In further experiments, using a method capable of determining 1  $\mu$ M (0.060 mg.), we have been unable to demonstrate, under a variety of conditions, any formation of steam-volatile (acetic or formic) acid by liver slices of diabetic cats. We used diabetic cats in which the initially high hepatic ketone formation had been reduced

\* Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *J. Biol. Chem.*, **132**, 423 (1940).

to low levels by intensive treatment with insulin in a period 2 to 3 hours before the preparation of the liver slices.

The experiments show that the prior insulin treatment tended to restore the fatty acid metabolism toward normal, as indicated by a diminished hepatic ketone formation. Nevertheless, there was no demonstrable formation of acetic acid. Likewise, in experiments with media containing fructose and fructose + insulin we found no acetic acid formation.

We conclude that the effect of insulin upon the fatty acid metabolism of the diabetic liver is to direct it along some pathway of partial oxidation not involving the formation of acetic (or formic) acid.

**Effect of Pancreatectomy on the Oxygen Uptake of Pigeon Muscle and Its Sensitivity to Insulin.** BY F. J. STARE AND C. A. BAUMANN. *From the Cancer Research Laboratory and the Department of Biochemistry, University of Wisconsin, Madison*

A marked difference was observed between the muscles of normal and depancreatized pigeons, both in the oxygen uptakes and the sensitivity to insulin. Insulin added to normal breast muscle *in vitro* increased respiration 20 per cent either in the presence or absence of other supplements. The sensitivity to insulin was increased to 70 per cent by removal of the pancreas. The optimum response was obtained 2 to 4 weeks after pancreatectomy. Insulin preparations inactivated by heat or alkali failed to stimulate muscle respiration.

Coccarboxylase increased the respiration of muscle from depancreatized pigeons; with muscle from normal pigeons an increase with coccarboxylase was observed only in the presence of other substances.

Glutamic acid, like certain other di(tri)-carboxylic acids, stimulated muscle respiration catalytically, and could replace these acids in sensitizing muscle to insulin *in vitro*.

**Modes of Combination of Acids and Ions with Wool Protein.** BY JACINTO STEINHARDT AND MILTON HARRIS. *From the Research Laboratory of the Textile Foundation at the National Bureau of Standards, Washington*

Previously reported experiments with HCl have indicated that

in combining with hydrogen ions, insoluble proteins such as wool keratin also form partially dissociated stoichiometric complexes with negative ions. This interpretation is now confirmed by titration curves obtained with other strong acids. Since the position of the curve for each acid with respect to the pH co-ordinate differs, the titration equilibrium is not determined by hydrogen ion dissociation constants alone. Measurements of the amounts of each of two acids taken up from mixtures support this conclusion, which may furnish a basis for understanding specific effects hitherto observed in kinetic and electrophoretic studies involving proteins.

Carboxylic acids differ from all others studied in that the amounts combined increase with increasing concentration without apparent limit. Much larger amounts of acetic, formic, glycolic, monochloroacetic, and dichloroacetic acids are bound than of HCl at any pH at which appreciable concentrations ( $> 0.01$  M) of the weak acid are present; this difference is greater the weaker the acid. The relation between amounts combined and amounts present is quantitatively described by assuming that, in addition to the ordinary acid-base equilibrium, part of the water of hydration of the protein is displaced by undissociated acid, in accordance with a simple partition law. Experiments with buffers in which the acid concentrations have been widely varied at a fixed pH support this assumption. By determining partition constants for a number of acids, large effects on the affinity of acid for protein of such factors as molecular size and specific substituents have been demonstrated.

#### Homocystine in Relation to the Synthesis of Mercapturic Acids.

By JAKOB A. STEKOL. *From the Department of Chemistry, Fordham University, New York*

In continuation of our work on the synthesis of mercapturic acids in animals, the rôle of homocystine in relation to the detoxication of bromobenzene and naphthalene in the rat was investigated.

Growing rats maintained on a 6 per cent casein diet ceased growing when bromobenzene or naphthalene was fed with the diet, and resumed growing when the bromobenzene or naphthalene diet was supplemented with homocystine. Rats fed daily a constant

amount of low casein diet supplemented with bromobenzene excreted just as much mercapturic acid as when homocystine was supplied to the same rats fed the same amount of diet. *dl*-Methionine or *l*-cystine, on the other hand, augmented the synthesis of the mercapturic acid.

These preliminary studies seem to indicate that the promotion of the growth of rats by homocystine on a diet containing bromobenzene or naphthalene is not necessarily an indication that homocystine augmented the detoxication processes to yield the corresponding mercapturic acids.

**On the Pasteur Enzyme in Retina.** BY KURT G. STERN, JOSEPH L. MELNICK, AND DELAFIELD DUBOIS. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

Pasteur discovered in 1861 that yeast fermentation is smaller in air than in carbon dioxide. In 1926, Warburg defined the Pasteur reaction as the inhibition of fermentation by respiration. However, Lipmann, Kempner, Laser, and others have shown that respiration and aerobic fermentation are independent processes and that the oxygen tension rather than the respiration is responsible for the phenomenon observed by Pasteur. The inability of oxygen to affect the fermentation in cell-free extracts indicates that in the cell there exists a substance which catalyzes the reaction between oxygen and the fermentation system. For this catalyst the name Pasteur enzyme is herewith proposed.

The enzyme may be electively inhibited by ethyl isocyanide (Warburg), by a lowering of the oxygen tension, and by suitable concentrations of CO; the latter inhibition is reversibly relieved by light (Laser).

The photochemical absorption spectrum of the CO compound of the Pasteur enzyme in rat retina shows a pattern similar to that of the respiratory ferment in yeast or in *Acetobacter*. However, the  $\gamma$  band in the blue shows a red shift of about 100 Å. and the  $\alpha$  band in the yellow shows a blue shift of about 140 Å. relative to the bands of the respiratory ferment.

*The Pasteur enzyme in retina appears to be a pheohemin protein like the respiratory enzyme in yeast or Acetobacter. It differs from*

*it with respect to its affinity for CO and O<sub>2</sub> as well as the position of the absorption bands of the CO compound.*

**Ascorbic Acid Oxidase.** BY ELMER STOTZ. *From the Biochemical Laboratory of the McLean Hospital, Waverley, Massachusetts, and the Department of Biological Chemistry, Harvard Medical School, Boston*

The activity of the copper-protein compound in cucumbers responsible for the aerobic oxidation of ascorbic acid can be measured manometrically at pH 6.0 (optimum pH) in the presence of glycine. The latter substance effectively inhibits the catalytic effect of inorganic copper impurities without affecting the activity of the enzyme. A unit of enzyme has been defined as the amount of enzyme which produces a rate of 100 c.mm. of O<sub>2</sub> per hour under specified conditions. The enzyme activity has been raised from 5 to 10 units per mg. of dry weight (crude cucumber extract) to approximately 850 units per mg. of dry weight, cucumber juice having been found to be the most active starting material. The procedure thus far involves water extraction, BaAc<sub>2</sub> clarification, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation, fractional heat denaturation, dialysis, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation. The activity of the enzyme is completely inhibited by small concentrations of Na diethyl dithiocarbamate and cyanide, and the final product contains 0.25 per cent Cu.

**Isolation of  $\gamma$ -Aminobutyric Acid from Liver.** BY YELLAPRAGADA SUBBAROW AND GEORGE H. HITCHINGS. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

In the course of concentrating a fraction of the liver which is active in promoting growth of rats,  $\gamma$ -aminobutyric acid, a homologue of  $\beta$ -alanine, has been isolated. The procedures involved in its isolation will be described.

**A Colorimetric Test for Methionine.** BY M. X. SULLIVAN AND TIMOTHY E. MCCARTHY. *From the Graduate School of Chemistry, Georgetown University, Washington*

When 0.3 to 1.0 cc. of 4 per cent aqueous solution of sodium nitroprusside is added to 5 cc. of a solution of methionine containing 0.2 mg. of methionine per cc. of water or 0.1 N HCl and the

solution is made alkaline with NaOH a yellow color develops. When acidified after 5 minutes standing, the color becomes strikingly red. Of the ordinary amino acids, histidine alone gives a red color on acidification. The other amino acids, cystine, cysteine, glycine, alanine, tyrosine, tryptophane, valine, arginine, etc., yield only a slight yellow color under this procedure. With acidification by mineral acids in the cold, creatine, creatinine, and various guanidines are negative. The interference by histidine can be eradicated in a number of ways, some of which will be detailed. The quantitative application of the reaction to the determination of methionine in proteins is in progress.

**Distribution of Water and Serum Electrolytes in Experimental Diabetes Mellitus.** BY F. WILLIAM SUNDERMAN AND F. C. DOHAN. *From the Department of Research Medicine, Pepper Laboratory, and Cox Institute, University of Pennsylvania, Philadelphia*

Measurements of serum volume, extracellular fluid, and concentrations of serum components were made in depancreatized dogs that had been permitted to go into ketosis following the withdrawal of insulin. Since the depancreatized animals suffered marked reduction in body weight, for comparison similar measurements were made following ketosis from simple fasting.

In the depancreatized dogs the average serum volume calculated in relation to the body weight was approximately 29 per cent greater during ketosis than during the control period, although the total serum volume was approximately 10 per cent less during ketosis than during the control period. In ketosis from fasting the total serum volume was decreased in proportion to the reduction in weight and within the limits of error of the measurements the serum volume in relation to body weight was unchanged.

The actual quantities of total base, chloride, bicarbonate, and protein in the circulating serum were decreased during ketosis in the depancreatized animals, although the amounts of these components, with the exception of bicarbonate, when expressed in relation to body weight were all increased.

The studies suggest that the dehydration observed in experimental diabetes mellitus induced by pancreatectomy in dogs is not made at the expense of serum volume or of the extracellular fluids but rather of the intracellular fluids.

**The Structure of Sphingomyelin.** BY S. J. THANNHAUSER AND MAX REICHEL. *From the Medical Clinic of the Boston Dispensary and Tufts College Medical School, Boston*

Sphingomyelins have been considered, according to the structural formula of Levene, as cholinephosphoric acid esters of sphingosine in which one hydroxyl group is free and the amino group is combined interchangeably with lignoceric, stearic, or palmitic acid.

The natural sphingomyelin occurring in brain, liver, spleen, and other organs is supposed to be a mixture of such substances, lignoceryl-, stearyl-, and palmitylsphingomyelin, with a free hydroxyl group on the sphingosine radical.

The enzymatic hydrolysis of sphingomyelin (spleen) with liver enzyme and purified pancreas lipase yielded the following. Splitting with liver enzyme resulted in formation of lignocerylsphingosine, choline, phosphoric acid, and palmitic acid. Hydrolysis with pancreatic lipase gave palmitic acid and lignocerylsphingomyelin. Saponification with diluted alkali at room temperature yielded the same products as in the case of pancreatic lipase. Neither palmitylsphingosine nor stearyl sphingosine has been isolated in any of the above procedures up to now. The yield of lignocerylsphingosine was in conformity with the presence of mainly one ceramide (lignocerylsphingosine) in sphingomyelin molecule (spleen).

Palmitic acid is split from sphingomyelin by pancreatic lipase and also by saponification with diluted alkali at room temperature. These two procedures do not split lignocerylsphingosine. This may be considered proof that the palmitic acid found in the sphingomyelin is not present in the form of a ceramide (NH—CO linkage) but as fatty acid ester linked to the free hydroxyl group of the sphingomyelin molecule.

Sphingomyelin occurs with a free hydroxyl group and as a sphingomyelin ester.

**Sulfonium Reactions of Methionine.** BY GERRIT TOENNIES.

*From the Lankenau Hospital Research Institute, Philadelphia*

Of the common natural amino acids methionine is, as a monosulfide, the only one capable of forming sulfonium derivatives:  $R'SR'' + R'''X \rightarrow R'R''R'''S^+ + X^-$  ( $X = \text{halogen or equivalent}$ )

lent group). We have obtained crystalline sulfonium salts by letting methyl iodide, methyl bromide, or allyl bromide react with *dl*-methionine, or its N-formyl or N-acetyl derivatives, in media composed of acetic and formic acid (the corresponding reactions with halogenacetic acids take place also in aqueous solution), removing solvent and excess halide by vacuum distillation, and treating the residue with methanol, acetone, or methyl acetate.

The new reaction appears of threefold interest. First, it provides a new hypothetical basis for bioreactions of methionine, such as its conversion to cysteine. Second, it opens new possibilities in the search for a rational procedure for the isolation of natural methionine. Third, it offers a new principle for the determination of methionine. Theoretical aspects of these three approaches are to be discussed and certain experimental results to be reported. Among the latter is evidence indicating that in proteins, dissolved in formic acid, the methionine grouping is available to react with bromoacetic acid, forming the sulfonium derivative which can be determined by the bromide ion liberated. The reaction is abolished by previous treatment of the formeous protein solution with hydrogen peroxide, obviously due to conversion of the methio group into the sulfoxide. To judge from the complete absence of a reaction between bromoacetic acid and glutathione in formic acid, protein thiol groups will not simulate methio groups under these conditions.

**The Metabolism of *dl*-Methionine Sulfoxide in the Dog.** By ROBERT W. VIRTUE AND MILDRED E. DOSTER-VIRTUE. *From the Department of Chemistry, University of Denver, Denver*

Fasting bile fistula dogs were fed 2.8 gm. of cholic acid daily. On the 3rd day of the fast an equivalent amount of *dl*-methionine sulfoxide was administered either orally or subcutaneously in addition to the cholic acid. Somewhat less than half the extra sulfur arising from the sulfoxide appeared in the sulfate fraction of the urine. Increased taurocholic acid excretion in the bile indicated that part of the sulfur of *dl*-methionine sulfoxide may give rise to taurine.

**Gonadotropic-Modifying Sera of Sheep Treated with Sheep Pituitary Extract.** By NELSON J. WADE AND PHILIP A. KATZ-



MAN. *From the Laboratories of Biology and Biological Chemistry, St. Louis University School of Medicine, St. Louis*

We have previously reported that the serum obtained from sheep which had been injected for a year with sheep pituitary extract continued to possess progonadotropic activity toward this extract in immature female rats. No evidence of inhibitory activity was obtained.

The present report contains the data obtained from the treatment of two sheep for an additional 7 months.

The sera of these animals after more than a year of the chronic injections first lost the property of augmenting the action of sheep pituitary extract on the rat's ovaries but retained the capacity to augment the uterine weight. This was followed by the loss of the progonadotropic effect as measured by both ovarian and uterine responses. The action of these sera was affected by the quantity of serum administered. Large amounts of serum of either animal produced a slight inhibition, whereas small quantities of the same serum of one of the sheep were augmentative. Salting-out with  $\text{Na}_2\text{SO}_4$  has not separated progonadotropic or anti-gonadotropic activity in any of the serum protein fractions.

The progonadotropic action of the serum of sheep treated with sheep pituitary extract is manifested in hypophysectomized as well as in normal female rats. This rules out the participation of the assay animal's hypophysis in this phenomenon. The progonadotropic activity can also be demonstrated in immature male rats. The failure of the serum *per se* to exert a gonadotropic effect in this animal indicates that the progonadotropic response is not due to luteinizing hormone.

**The Metabolism of Glutathione Studied with  $\text{N}^{15}$ .** By HEINRICH WAELSCH AND D. RITTENBERG. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

The three amino acids of glutathione (GSH) are the same as are used by the mammalian organism for conjugation with compounds other than amino acids (formation of bile acids, detoxication). If GSH functions as amino acid donor for such

conjugations, the replacement of its amino acids must be fast. The rate of turnover of GSH was investigated with glycine marked with  $N^{15}$ .

A method is described for the isolation of GSH as the copper compound from single organs of rats and rabbits. From liver the yields (maximum 101 mg. from a rabbit liver) averaged 60 per cent of the value obtained by iodine titration. When rats were given 75 mg. of marked glycine, the GSH isolated from the liver after 2 and 5 hours contained a concentration of isotope corresponding to 9 and 8 per cent of that in the administered glycine. Under the assumption that all isotope was in the glycine moiety of the GSH, about one-fourth of its glycine was derived from the administered amino acid. By contrast only about one-sixtieth of the glycine of the liver proteins came from administered glycine. The distribution of  $N^{15}$  in GSH is being established by isolation and analysis of the components of the liver GSH.

The rapid turnover of GSH may be the first indication that peptides and particularly GSH are intermediates in transferring amino acids in protein metabolism.

**Lipid Metabolism in Brain during Myelination.** BY HEINRICH WAELSCH AND WARREN M. SPERRY. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

The deposition of lipids in the rat brain was studied during myelination. Heavy water was injected into mother rats and their young at 15, 26, and 36 days after birth and the drinking water was enriched with deuterium. The heavy water régime was continued for 4 days, after which the deuterium content of the unsaponifiable lipids and fatty acids of brain, liver, intestine, and remaining carcass of the mother rats and their young was determined.

Unsaponifiable lipids and fatty acids are deposited in brain at a rapid rate from the 15th to 19th day of extrauterine life. The rate is considerably less from the 26th to the 30th day, and still lower from the 36th to the 40th day. If allowance is made for turnover comparable with that found in adult rats, no marked

differences in the rates of deposit of the unsaponifiable and fatty acid fractions are evident.

The data indicate that lipids deposited between the 15th and 19th day of life are synthesized in the brain itself.

The relationships among rates of lipid metabolism in the liver, intestine, and remaining carcass were in general the same as were found in adult rats, but in most instances the deuterium uptake was considerably higher in the young than in the adult rats.

Determinations on mother rats agreed closely on most points with the findings in adult male rats.\*

**Effect of Cysteine on Ascarids.** BY A. WALTI. *From the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey*

When living ascarids are placed in contact with a proteolytic enzyme of the ficin type, the worms are digested.

It was observed that this digestive property of the proteolytic enzyme is greatly increased by the presence of cysteine. Thus, 3 to 7 times the amount of commercial papain is necessary to produce the same digestive effects on helminths as when cysteine is added as an activator. Similar results were observed with bromelin.

The cysteine used in these experiments had no effect on the ascarids in the amounts used, as shown by control experiments. However, when the amount of cysteine was increased several fold and the time of interaction lengthened from the customary 2.5 to 18 hours, marked effects on the ascarids were observed which resembled some stage in the enzymatic digestion of the helminths. Alanine was used as a control in parallel experiments.

**A Disturbed Carbohydrate Metabolism in Rats Maintained More Than a Year on a Fat-Deficient Diet.** BY LAURENCE G. WESSON. *From the Forsyth Dental Infirmary, Boston*

By the use of an improved, fat-deficient diet, rats were obtained that were less emaciated and that lived longer than on the fat-deficient diets previously used. Glucose tolerance and dextrin R.Q. tests indicated a disturbed carbohydrate metabolism after 12 to 15 months on this diet. These tests, some of them carried out

\* See accompanying abstract by Sperry and Waelsch.

with and without the administration of insulin and with and without the addition of small amounts of a natural fat to the diet, indicate that possibly this disturbance is caused by the simultaneous lack of a carbohydrate metabolism factor of natural fats and a disturbance in the functioning of the pancreatic islets.

**An Improved Microtitration Method for the Determination of Carbon Dioxide in Blood and Other Fluids.** BY EDWARD S. WEST, BERT E. CHRISTENSEN, AND ROBERT E. RINEHART.  
*From the Department of Biochemistry, University of Oregon Medical School, Portland*

Christensen has devised an apparatus and method for the microdetermination of carbon and hydrogen in organic compounds by a wet combustion process in which the carbon dioxide evolved from the reaction chamber is aspirated into an evacuated flask containing standard barium hydroxide. The procedure has been adapted to the determination of carbon dioxide in blood and other fluids. The apparatus is easily constructed, simple to operate, and yields results comparable to those obtained by the Van Slyke manometric method.

**Effect of the Oral Administration of Benzpyrene on the Growth of the Rat Ingesting a Diet Low in Lysine.** BY JULIUS WHITE.  
*From the National Cancer Institute, United States Public Health Service, Bethesda, Maryland*

It has been previously shown that the oral administration of benzpyrene to young growing rats on a relative low protein basal diet, resulted in a retardation of growth. Furthermore the addition of *l*-cystine or *dl*-methionine to the diet still containing the hydrocarbon resulted in a stimulation in growth. It appeared therefore that the growth-inhibitory substance exerted its effects by the production of a specific deficiency in the sulfur-containing amino acids, probably through the requirements of the organism for organic sulfur for detoxication mechanisms in the form of cystine and methionine. In order to determine whether other essential amino acids could detoxify ingested carcinogens, young growing rats were placed on a diet low in lysine but adequate for normal growth. The addition of benzpyrene (110 mg. per 100 gm. of diet) produced a marked retardation in growth. The

effect of the addition of lysine, cystine, or methionine to the diet containing the hydrocarbon on the growth of the rat will be presented.

**Effect of the Oral Administration of Dimethylaminoazobenzene (Butter Yellow) on the Growth of the Rat.** BY JULIUS WHITE.  
*From the National Cancer Institute, United States Public Health Service, Bethesda, Maryland*

The growth-inhibiting effect produced by the oral administration of dimethylaminoazobenzene has been demonstrated on young growing rats by the incorporation of the dye in a relatively low protein basal diet. The addition of 60 mg. of the butter yellow to 100 gm. of basal diet resulted in a change in growth rate from an average of 1.4 to 2.0 on the basal diet alone to an average of 0.3 to 0.6 gm. The addition of either *l*-cystine (500 mg.) or *dl*-methionine (500 mg.) to each 100 gm. of basal diet still containing the hydrocarbon resulted in a stimulation of growth with a subsequent daily weight increase which approximated that observed in rats ingesting the basal diet alone. On the other hand, supplements of either glycine, taurine, or sodium sulfate did not stimulate growth when these substances were added to a diet containing the butter yellow. The effect of the addition of lysine to the diet containing the dye will be discussed.

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